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Vitamin B_{12} Symposium

The Disappearance of Intravenously Administered Vitamin B_{12}

Studies in Normal Subjects and in Patients with Pernicious Anemia

SOLOMON ESTREN, M.D.,* EUGENE A. BRODY M.D.† AND LOUIS R. WASSERMAN, M.D.‡

THE STUDIES to be described were designed to follow the fate of a small dose of radioactive vitamin B_{12} after its intravenous injection into "normal" subjects and patients with a variety of disorders. Following such injection, it is possible to plot the rate of disappearance of radioactivity from the plasma, the rates of uptake in the liver and the bone marrow, and the excretion in the urine. The pattern of disappearance was found to differ in certain disease states. We shall describe this pattern in the normal subject, in patients with chronic myelocytic leukemia and in patients with pernicious anemia in relapse or in remission.

From the Department of Hematology, The Mount Sinai Hospital, New York, New York.

* Associate Attending Hematologist; † Assistant Attending Hematologist; ‡ Director, Department of Hematology.

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MATERIAL AND METHODS

A standard dose of 0.5 μg. (rarely, 1.0 μg.) of Co^{58}-vitamin B_{12} or Co^{60}-vitamin B_{12} was rapidly injected intravenously into a subject. Specimens of blood were collected in heparin at frequent intervals after the injection: one, three, five, fifteen, thirty and sixty minutes, and then at two, six or eight hours, and twenty-four hours. At the same time, *in vivo* radioactivity was measured over the liver and over the sacral bone marrow. The radioactivity present in an aliquot of plasma at each point in time was measured in a scintillation counter and converted to total plasma radioactivity by estimating the plasma volume of the patient, and this total plasma radioactivity was then expressed as a percentage of the injected dose.

The radioactivity excreted in the twenty-four-hour urine sample following the injection was similarly determined and was also ex-

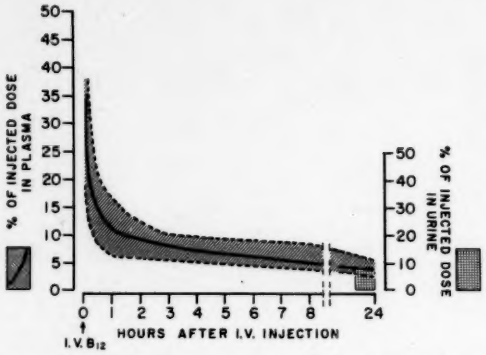

FIG. 1. Pattern of disappearance following the intravenous administration of radiocobalt vitamin B_{12} in the normal subject.

pressed as a percentage of the injected dose. No radioactivity appeared in the stool or in the circulating red cells. The simultaneous *in vivo* counting showed slight but definite radioactivity over the sacral marrow, and marked radioactivity over the liver. We have not, however, found a satisfactory method of quantitating these *in vivo* counts in terms of the injected dose.

In addition, microbiologic assay of vitamin B_{12} was performed on each specimen of plasma by means of Euglena gracilis.

RESULTS

The pattern of disappearance in the normal subject* is shown in Figure 1. If the first specimen of plasma is obtained early enough, within one minute, the radioactivity remaining in the plasma may be as high as fifty to sixty per cent of the injected dose. Within three to five minutes, seventy per cent of the injected radioactivity has already disappeared from the plasma. In one hour the residual radioactivity is ten to twelve per cent; in six to eight hours, between five and ten per cent; and in twenty-four hours, five per cent or less. The twenty-four-hour urinary excretion never exceeds five to eight per cent of the injected dose. Thus, after twenty-four hours, five per cent of the dose is still in the plasma, five per cent has appeared in the urine, and ninety per cent is as yet unaccounted for.

* Our normal subjects had no evidence of hematologic disease, hepatic disease or renal disease.

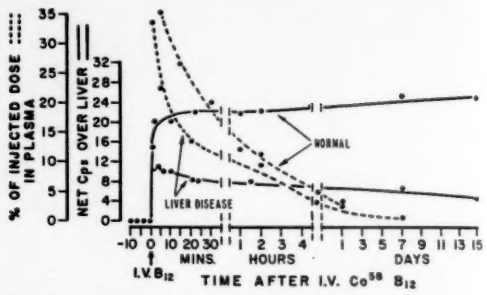

FIG. 2. Plasma disappearance and hepatic uptake of injected radiocobalt vitamin B_{12}.

Much of this ninety per cent can be assumed to be present in the liver. Thus, in Figure 2, disappearance of radioactive vitamin B_{12} from the plasma is plotted together with hepatic uptake of radioactive vitamin B_{12} (expressed as net counts per second) in a normal subject and a patient with severe liver disease. As seen by the normal curves, there is an instantaneous rise in hepatic radioactivity, i.e., in the uptake of vitamin B_{12} from the plasma by the liver. That the persistence of this rise is related at least in part to the ability of the liver to bind vitamin B_{12} normally, is suggested by the curve of the patient in Figure 2. This patient had hepatic disease and showed considerably less uptake of radioactivity than did the normal subject.

Quantitation of the percentage of the injected dose taken up by the liver and the bone marrow has not been possible. Postmortem studies have suggested, however, that approximately fifty per cent of the injected dose is taken up by the liver, with smaller amounts by kidney, pituitary, bone marrow and other tissues.

Simultaneous microbiologic assay in normal persons (Figure 3 is typical) similarly shows that, within the first few minutes, there is a rapid rise of plasma vitamin B_{12}, and that this rise is accounted for by a rise in the bound vitamin. The total vitamin B_{12} level then falls rapidly to the preinjection level, the fall in the bound fraction paralleling the fall in total vitamin B_{12}. The microbiologic curve parallels the radioactive disappearance curve.

In attempting to determine why the curve of vitamin B_{12} disappearance has its particular

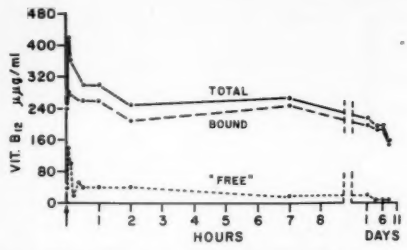

FIG 3. Disappearance of intravenously injected vitamin B_{12} in the normal subject, measured by microbiologic assay. Arrow indicates time of injection.

shape, we studied an artificial situation in which a normal person was given an injection of 1,000 μg. of vitamin B_{12} intramuscularly just prior to the intravenous injection of radiocobalt vitamin B_{12}. The resulting curve is shown in Figure 4. This curve is displaced downward as compared with the normal curve and, at the same time, the urinary excretion of radioactivity, which is normally 5 to 8 per cent, becomes 75 per cent. Presaturation with vitamin B_{12}, in other words, presumably so saturates the serum and other body proteins that, when additional (radioactive) vitamin B_{12} is injected, little additional (radioactive) vitamin B_{12} can be accepted by these proteins and plasma radioactivity rapidly disappears from the blood because of its ready excretion in the urine.

In contrast to this artificial situation is the curve of the patient with chronic myelocytic leukemia. This curve is raised above the normal (Fig. 5). In other words, at any given time, there is more residual radioactivity (i.e., vitamin B_{12}) in the plasma of patients with this disorder than in the normal person. This fact cannot be accounted for by the radioactivity excreted in the urine, which is essentially the same as in the normal subject. The curve is unique, is apparently found only in patients with chronic myelocytic leukemia and is related to the proteins in this disease. Although chemical and ordinary electrophoretic methods have shown no difference between the plasma proteins of normal subjects and those of patients with chronic myelocytic leukemia, special studies (in which the amount of vitamin B_{12} present in each protein fraction was determined) have shown increased binding of vita-

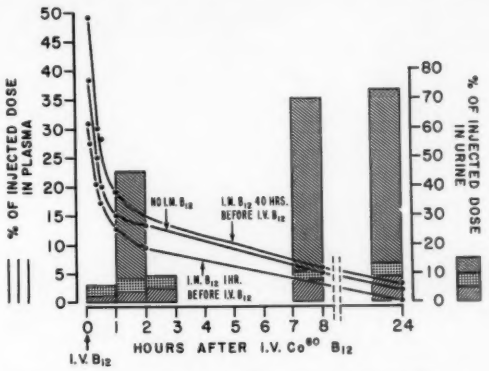
% OF INJECTED DOSE IN PLASMA
% OF INJECTED DOSE IN URINE
NO I.M. B12
I.M. B12 40 HRS. BEFORE I.V. B12
I.M. B12 1 HR. BEFORE I.V. B12
I.V. B12
HOURS AFTER I.V. Co60 B12

FIG 4. Effect of presaturation with 1,000 μg. vitamin B_{12} (intramuscularly) on the disappearance curve in the normal subject. The highest urinary excretion (diagonal shading to the right) corresponds to presaturation one hour before the intravenous dose. See text.

min B_{12} to the alpha-globulins in the leukemic plasma. Apparently, an abnormal protein in the alpha-globulin region is present in patients with this disease, which has the property of binding increased amounts of vitamin B_{12}.[1]

This concept is confirmed by the extremely high serum vitamin B_{12} levels known to be present in this disease. It is further confirmed when the test dose of 0.5 μg. of radiocobalt vitamin B_{12} is incubated with plasma from a patient with chronic myelocytic leukemia prior to its injection into a normal subject (Fig. 6). The resulting disappearance curve is not normal but resembles that seen in the leukemic patient. This result is found only with chronic myelocytic sera, and is not found after preincu-

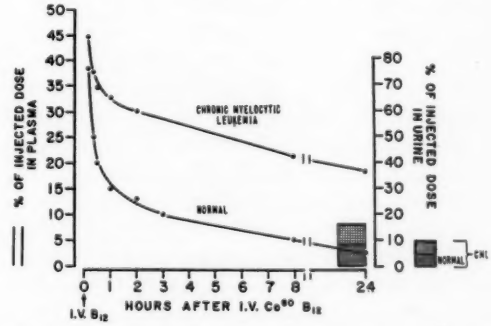

FIG. 5. Disappearance of injected radiocobalt vitamin B_{12} in the patient with chronic myelocytic leukemia.

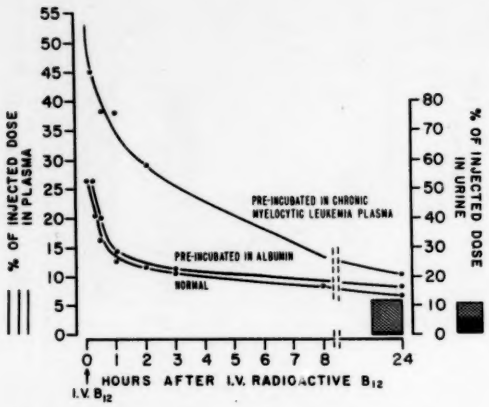
% OF INJECTED DOSE IN PLASMA
% OF INJECTED DOSE IN URINE
PRE-INCUBATED IN CHRONIC MYELOCYTIC LEUKEMIA PLASMA
PRE-INCUBATED IN ALBUMIN
NORMAL
HOURS AFTER I.V. RADIOACTIVE B_{12}
I.V. B_{12}

FIG. 6. Effect of preincubation with radiocobalt vitamin B_{12} in plasma of patients with chronic myelocytic leukemia, on the disappearance curve after subsequent injection into the normal subject.

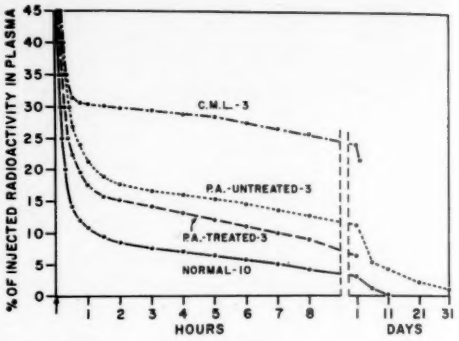

FIG. 7. Disappearance of injected radiocobalt vitamin B_{12} in patients with pernicious anemia in relapse and in remission. Arrow indicates time of injection.

bation with other sera (e.g., normal, lymphosarcoma) or with human serum albumin.

With this background information we became interested in studying the disappearance test in patients with pernicious anemia. We explained the normal curve by initial binding of injected vitamin B_{12} by serum protein, followed by rapid uptake of the plasma vitamin B_{12} by the liver and other tissues. We explained the presaturated curve, with its artificially rapid disappearance, on the basis that the serum (and tissue) proteins were already saturated by vitamin B_{12} before the radioactive material reached them, so that they could not accept the additional vitamin B_{12}. We explained the leukemic curve, with its abnormally slow disappearance, by the increased capacity of the serum proteins in this disease to bind vitamin B_{12}.[2]

In patients with pernicious anemia, the serum proteins are normal in their binding ability, and the tissues, which are depleted of vitamin B_{12}, might be expected *a priori* to take up avidly large amounts of the injected vitamin. We therefore anticipated that the disappearance of injected vitamin B_{12} might be more rapid than normal, so that the curve would be displaced downward. The actual results, however, were entirely different, and demanded more profound analysis. Thus (Fig. 7), patients with untreated pernicious anemia showed a displacement of the curve upward as compared with the normal subjects; i.e., disappearance was slower than in the normal person, although not as slow as in patients with chronic myelocytic leukemia. Even more unexpected was the finding that patients with pernicious anemia in remission still showed a disappearance curve displaced upward from the normal curve, i.e., disappearance was less rapid than in the normal subjects, although not as slow as in patients with pernicious anemia in relapse.

Studies of liver and urine function failed to explain these findings: Hepatic uptake of radioactivity did not differ from that in the normal subject, and urinary radioactivity was the same in patients with pernicious anemia as in the normal subject. Simultaneous microbiologic studies showed that, as in the normal subject, there was an initial rise in total vitamin B_{12}, due to a rise in bound vitamin B_{12}, and that, as the total vitamin B_{12} level fell, the bound material closely paralleled its fall.

We were therefore unable to explain why the disappearance of injected vitamin B_{12} from the plasma was abnormally rapid in patients with pernicious anemia in relapse and, more significant, why it remained abnormally rapid even after adequate vitamin B_{12} therapy had caused complete clinical and hematologic remission. On purely theoretical grounds, one might list those factors which regulate the disappearance of injected vitamin B_{12} from the normal plasma (Table I). These would include loss to the outside (urine) and loss to the inside

TABLE I

Factors Which May Regulate Disappearance of Injected Vitamin B_{12} from Normal Plasma

Loss via
Urinary excretion
Tissue (liver) uptake
Mechanism of tissue uptake
Ability of liver to retain vitamin B_{12} (in hepatic disease, liver binding falls, urine vitamin B_{12} rises)
? serum factors which transfer vitamin B_{12} from serum protein to liver protein

(uptake by body tissues, especially the liver). The functional capacity of the liver is related to the curve since, in a number of patients with hepatic disease, vitamin B_{12} uptake by the liver seemed to be diminished. In addition, however, the question arises as to whether there might also be substances in the serum which aid the transfer of vitamin B_{12} from its location in the serum (alpha) globulins to the beta-1 globulin which is said to bind vitamin B_{12} within the liver.

Table II lists factors which might help explain the two groups of patients with diminished rates of plasma disappearance, chronic myelocytic leukemia and pernicious anemia. In patients with chronic myelocytic leukemia, the serum proteins bind more vitamin B_{12} than do serum proteins and liver protein in normal subjects. This fact adequately explains the resultng curve, although the possibility of diminution of serum "B_{12}-transferase," in addition to the increased binding by serum proteins, theoretically exists.

In patients with pernicious anemia in remission, however, it is known that the total serum B_{12} and the proportion of the bound vitamin are the same as in the normal person. In addition, it is known that the capacity of patients with pernicious anemia serum to bind vitamin B_{12} is the same as that of normal subjects. Although the capacity of the serum in patients with pernicious anemia in relapse to bind injected vitamin B_{12} is increased above normal by virtue of the fact that the circulating serum vitamin B_{12} is virtually zero, this state no longer holds when remission has occurred, so that the abnormally slow disappearance in patients with pernicious anemia

TABLE II

Factors Which May Decrease Disappearance of Injected Vitamin B_{12} from the Plasma

Chronic myelocytic leukemia
Increased binding by serum proteins
Serum proteins bind B_{12} more than liver proteins, or
Lack of "B_{12}-transferase" as well as increased binding by serum proteins
Pernicious anemia
Total serum vitamin B_{12} and vitamin B_{12}-binding capacity are same as in normal
Hence
Binding by serum proteins is greater than normal, or
Binding by liver proteins is less than normal, or
"B_{12}-transferase" is lacking

in remission cannot be explained by this "unbound binding capacity." The slow disappearance thus is not the result of increased binding of the injected vitamin B_{12}, since there is no increased binding. It might be explicable by a diminished ability of liver protein to accept vitamin B_{12}, but there is no evidence for any such diminished ability. The slow disappearance in patients with pernicious anemia in remission might also be explained by a lack of the hypothetical "serum B_{12}-transferase" mentioned before.

At this point, all discussion becomes quite theoretical, since we do not have data to support any of this speculation. It is generally accepted that the dietary vitamin B_{12} which goes into the lumen of the gastrointestinal tract requires intrinsic factor to allow its transfer to the intestinal mucosa: this intrinsic factor is lacking in patients with pernicious anemia, whether in relapse or in remission. How vitamin B_{12} passes from intestinal mucosa to the plasma is not, to our knowledge, known. This passage might be mediated by an enzyme, or perhaps by some substance such as intrinsic factor.[3] How, once vitamin B_{12} is present within the plasma and attached to serum globulin, it then passes from the plasma to the tissue (liver) proteins, is also unknown. This step might also be due to the same intrinsic factor in the blood, or to a transferring enzyme of another kind. The patient with pernicious anemia, known to lack intrinsic factor within

the gastrointestinal tract, would then be expected to lack intrinsic factor within his serum.

We should like to suggest that the abnormal pattern of disappearance of vitamin B_{12} from the plasma following its intravenous injection into patients with pernicious anemia can best be explained, especially in complete remission, by the postulate of "serum B_{12}-transferase" which, present in normal amounts in normal sera, is diminished or absent in patients with pernicious anemia, whether in relapse or in remission.

Whether this postulate will withstand the test of further experimentation remains, of course, to be determined.

SUMMARY

The fate of radioactive vitamin B_{12} following its intravenous injection was determined in normal subjects and in patients with chronic myelocytic leukemia and pernicious anemia.

The resulting plasma disappearance curves were abnormal patients with chronic myelocytic leukemia and pernicious anemia.

The abnormally slow plasma clearance of vitamin B_{12} found in pernicious anemia persisted even in patients in complete hematologic and clinical remission.

The concept of a circulating "serum B_{12}-transferase" is suggested to explain these findings.

REFERENCES

1. Heinrich, H. C. and Erdmann-Oehlecker, S. Der Vitamin B_{12}-Stoffwechsel bei Hämoblastosen. II. Die intravitale Bindung (Transport) der B_{12}-Vitamine an die Serumproteinfraktionen bei Hämoblastosen. *Clin. chim. acta*, 1: 311, 1956.

2. Raccuglia, G., and Sacks, M. S. Vitamin B_{12} binding capacity of normal and leukemic sera. *J. Lab. & Clin. Med.*, 50: 69, 1957.

3. Herbert, V. and Spaet, T. H. Distribution of 'intrinsic factor' activity. *Am. J. Physiol.*, 195: 194, 1958.

Vitamin B_{12} in Pregnancy and the Puerperium

LOUIS LOWENSTEIN, M.D., MADELEINE LALONDE, EARL B. DESCHENES AND LORNE SHAPIRO, M.D.

In a previous study of nineteen cases of megaloblastic anemia in pregnant and puerperal women at the Royal Victoria Hospital, it was concluded that, although this group of anemias is not homogeneous, in many cases the anemia was probably related to nutritional deficiency.[1] In all patients to whom folic acid or folinic acid was given, the hematologic response was satisfactory. This was also true in four of eight patients who were given vitamin B_{12}.

In contrast to earlier reports, similar experiences with vitamin B_{12} have recently been documented from non-tropical areas. In view of these findings, a study was undertaken to determine the serum vitamin B_{12} level in normal pregnant women and in puerperal women as well as the urinary excretion of folic acid over a six-hour period when given orally, subcutaneously or intramuscularly in doses of 5 mg.

In 1954, Heinrich reported a decrease in the serum and urinary vitamin B_{12} levels in normal pregnant women.[2] Since his original publication, several observers, including our group in Canada, have confirmed these results.[3–9] The present paper is concerned with a summary of our studies during the past four years on the following: (1) the serum vitamin B_{12} level in normal pregnant and puerperal women; (2) the simultaneous observations of the serum vitamin B_{12} level in mothers at delivery and in the cord blood of their infants; (3) the preliminary results of observations of the binding capacity of serum for the vitamin in normal pregnant women and in normal* women; (4) the folic acid urinary excretion in normal pregnant women and in normal women after oral or parenteral administration of 5 mg. of folic acid; and (5) some serum estimations of vitamin B_{12} concentration in women with the anemias of pregnancy.

From the Hematology Service, Department of Medicine, Royal Victoria Hospital, and University Clinic, McGill University, Montreal, Canada.

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This study was aided by Canadian Federal-Provincial Research Grant No. 604-7-49.

MATERIAL AND METHODS

Vitamin B_{12} levels were determined in the blood serums by microbiologic assay with Lactobacillus leishmanii using the method of Skeggs et al.[10] The basal medium was made with commercial dehydrated medium (Difco®), the innoculum was prepared and the test organism was maintained according to the U.S.P. XV. Blood samples were taken under sterile conditions, allowed to clot, centrifuged and stored at −20°C. until assay. One volume of the serum was diluted to 15 volumes of water and duplicate aliquots were used for the determination. Triplicate aliquots of 1.25, 2 and 4 ml. were used for the determination. Triplicate aliquots of 1.25, 2.5 and 5 ml. of a solution of crystalline cyanocobalamin containing 20 μμg./ml. were used as a standard for each set of determinations. The serum vitamin B_{12} concentrations were calculated by the slope ratio method described by Bliss.[11]

* Throughout this paper, "normal" women refers to normal women of childbearing age who were not pregnant.

TABLE I

Serum Vitamin B_{12} Levels in Normal Subjects

Subject	Patients (no.)	Mean Serum Vitamin B_{12} Levels ($\mu\mu$g./ml.)	Standard Deviation ($\mu\mu$g./ml.)	Range (μgg./ml.)
Normal subjects (previous study) mixed ages and sexes	101	575	287	234–1,694
Normal women	49	560	244	120–1,010

Folic acid excretion tests were performed by obtaining a control sample of urine, then administering 5 mg. of folic acid orally with 400 ml. of water. All urine voided during a six-hour period was collected, and the excretion of folic acid was determined microbiologically according to the method of Teply and Elvehjem.[12] The results were considered invalid if less than 300 ml. of urine was voided during the six-hour test period.

A complete dietary history of each patient was obtained. Hemoglobin, packed cell volume, red cell counts, total and differential leukocyte counts and morphologic study of the red cells were performed at the time the serum vitamin B_{12} levels were determined.

The *in vitro* binding capacity of serum was determined with cobalt^{58}-vitamin B_{12}, using simultaneously a modification of the ultrafiltration method of Gregory[13,14] and a modification of the charcoal adsorption method of Miller.[15,16] Initial samples of serum were assayed microbiologically using L. leishmanii for the vitamin B_{12} content, and subsequent samples were assayed both microbiologically and radiometrically.

RESULTS

The results of two series of studies in normal patients are presented in Table I. In the first group, consisting of 101 subjects of both sexes and mixed ages, the mean serum vitamin B_{12} level was 575 $\mu\mu$g./ml. In the second group, which consisted of normal women of childbearing age, the mean serum vitamin B_{12} level was 560 $\mu\mu$g./ml. In both groups, the standard deviation was large. These results are similar to those obtained by Boger and his associates[17] in a group of 528 subjects using the L. leishmanii method.

TABLE II

Serum Vitamin B_{12} Levels in Normal Pregnant Women

Trimester	Observations	Mean ($\mu\mu$g./ml.)	Standard Deviation ($\mu\mu$g./ml.)	Range ($\mu\mu$g./ml.)
First	30	343	173	123–867
Second	59	319	137	110–516
Third	148	300	171	47–1,284

Table II shows the serum vitamin B_{12} concentration in normal pregnant women during the three trimesters. Thirty women studied during the first trimester were also observed during the second and third trimesters. The serum levels were determined in an additional forty-seven women in both the second and third trimesters and in still another group of seventy women during the third trimester. Since these studies were made, a similar study was carried out in another group of 300 patients and the results were essentially the same. Of interest is the fact that the mean serum vitamin B_{12} concentration decreases progressively during pregnancy. The deviation of the mean in pregnant women was not as great as that in normal subjects.

Even in the first trimester there is a considerable decrease of the serum level as compared with the level in normal subjects. The serum level decreased further during pregnancy and reached its minimum during the third trimester. An analysis of variance for all observations in these classes shows that the depression of the serum vitamin B_{12} concentration is highly significant ($P = 0.001$) over the duration of the pregnancy expressed in trimesters. The deviation from linearity is also significant ($P = 0.01$) and indicates that this regression cannot be completely represented by a straight line. A regression coefficient showed that the decrease in vitamin B_{12} was approximately 75 $\mu\mu$g./ml. per trimester. The actual distribution was skewed to the right and 1 standard deviation includes approximately 80 per cent of the observations. All values observed were included in the analysis.

TABLE III

Hemoglobin Levels in Normal and Pregnant Women

Subjects	Observations (no.)	Mean Hemoglobin Levels (gm./100 ml.)	Standard Deviation (gm./100 ml.)	Range (gm./100 ml.)
Normal women	51	13.6	1.0	10.6–16.4
Pregnant women				
Second trimester	32	12.32	1.06	9.9–14.2
Third trimester	103	12.35	0.99	9.8–15.1

In normal subjects, values of less than 200 μμg./ml. were uncommon. In the pregnant women, serum levels of less than 200 μμg./ml. were exhibited by six of thirty-four patients studied during the first trimester, by nine of fifty-nine patients studied during the second trimester, and by forty-eight of 252 patients studied during the third trimester. Nineteen patients exhibited levels between 100 and 149 μμg./ml. and in eight patients the serum vitamin B_{12} concentration was less than 100 μμg./ml. Using this method of assay, patients with pernicious anemia in relapse may exhibit serum vitamin B_{12} levels of 100 to 150 μμg./ml. and the critical level is probably somewhat higher than the 100 μμg./ml. or less which has been found using the Euglena gracilis method.[31]

Table III depicts the hemoglobin levels of the normal women of childbearing age, and of the patients in whom the serum vitamin B_{12} levels were obtained during the second and third trimesters. Statistical analysis of these data showed that there is less than one change in one thousand of the group of patients in the second and third trimesters belonging to the same population as those in the normal groups ($T = 5.75$ for second trimester of normal women; $T = 4.25$ for third timester of normal women). On the other hand, there is more than a 90 per cent probability that the subjects in the second and third trimesters belonged to the same type of population ($T = 0.1078$).

From Table IV, a similar type of analysis was made for the packed cell volume and statistical results obtained were essentially the same. The t test showed that the samples of

TABLE IV

Packed Cell Volume in Normal and Pregnant Women

Subjects	Observations (no.)	Mean Packed Cell Volume (%)	Standard Deviation (%)	Range (%)
Normal women	51	42.14	3.5	35–49
Pregnant women				
Second trimester	32	37.81	3.03	30–42
Third trimester	103	37.80	3.8	30–45

the two normal groups came from the same type of population and the Fisher F test showed that there was no significant difference in the variance of the two normal groups previously shown (Table I).

Studies of the urinary excretion of folic acid were performed on 55 normal pregnant women in the third trimester after the oral administration of 5 mg. of folic acid, Table V; similar

TABLE V

Folic Acid Balance Studies in Normal Pregnant Women and in Normal Men and Women

Subjects	Route of Administration	Patients (no.)	Mean Excretion (%)	Range (%)
A. Per Cent of 5 Mg. Dose of Folic Acid Excreted in Urine in Six Hours				
Pregnant women, third trimester.	Orally	55	24.8	4.8–58.0
Normal women	Orally	17	28.4	8.2–55.8
Pregnant women, third trimester.	Intramuscularly	36	41.5	16.5–94.0
Men and normal women	Intramuscularly	12	42.5	13.0–86.0

Subjects	Patients* (no.)	Ratio†	Standard Deviation	Standard Error
B. Ratio of Folic Acid Excreted in Urine Six Hours After Administration				
Men and normal women	10	0.56	0.21	0.07
Pregnant women, third trimester.	20	0.52	0.19	0.04

* Patients were included in this study only if the volume of both six-hour urine samples exceeded 300 ml. and were within 15 per cent of each other.

† Folic acid excreted after oral dose/Folic acid excreted after injection

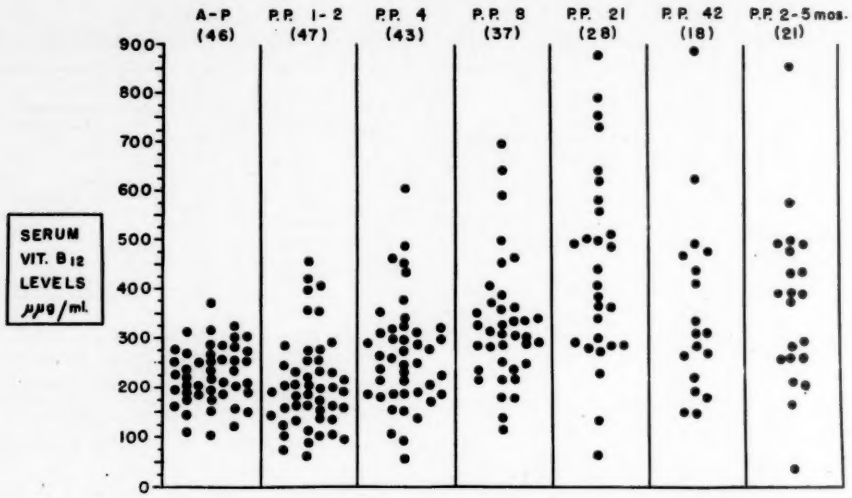

FIG. 1. Serum vitamin B_{12} studies in antepartum and postpartum patients.

studies were repeated after the intramuscular injection of 5 mg. of folic acid in 36 of these women. The results were compared with those of identical studies made in seventeen normal men and normal women of childbearing age. Statistical analysis showed no significant difference in the folic acid excretion of pregnant women during the third trimester after oral or intramuscular administration of folic acid as compared with those of the normal control subjects.

These negative results do not exclude the possibility that folic acid metabolism may differ in pregnant women as compared with normal women. Such differences, if they exist, might be demonstrated by more sensitive methods.

Table VI shows the serum vitamin B_{12} values

TABLE VI

Serum Vitamin B_{12} Levels of Normal Pregnant Women Before and After Delivery

Time	Observations (no.)	Mean (μμg./ml.)
Antepartum	45	220
Postpartum		
Days 1 and 2	47	208
Day 4	43	266
Day 8	34	316
Day 21	25	429

of normal pregnant women studied shortly before delivery and serially during the puerperium. An analysis of variance of these data was made and showed that the regression of serum vitamin B_{12} concentration was highly significant ($P = 0.001$) during the postpartum period. The deviation from linearity again was significant ($P = 0.01$) and indicates that this regression cannot be completely represented by a straight line. The regression coefficient shows that serum vitamin B_{12} concentration increased by 4.45 μμg./ml. per postpartum day. In examining the individual data, the decrease of values during the first and second postpartum days as compared to the antepartum values was often more marked than is represented by the mean.

Figure 1 is a scattergram of these values. Unfortunately, the number of patients cooperating at six or more weeks postpartum was too few to permit accurate statistical analysis. However, the serum vitamin B_{12} concentrations were still lower than those of normal subjects in a number of patients observed at these postpartum periods.

In Table VII are tabulated the serum vitamin B_{12} values of blood from 100 mothers and the cord blood of their infants. The mothers were studied within a few hours of delivery in thirty-two instances, and within

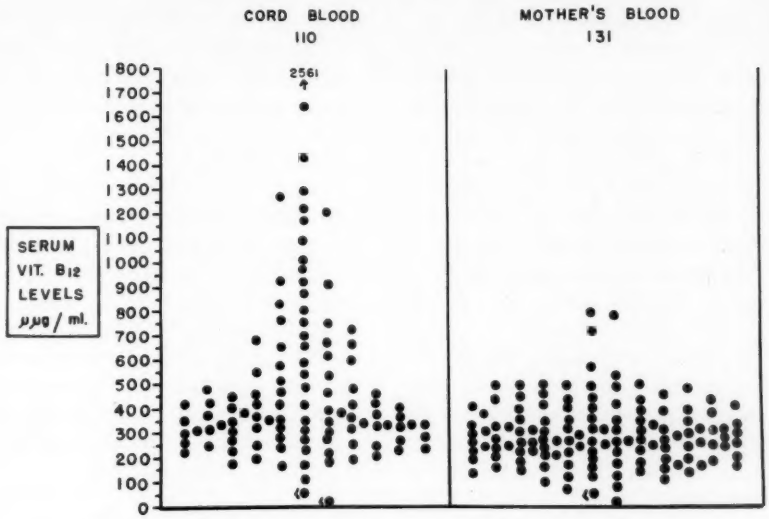

FIG. 2. Serum vitamin B_{12} levels in cord blood and mother's blood.

twelve to seventy-two hours postpartum in 133 cases. Repeat studies of some of the mothers in that period are included in the tabulation.

The mean values of the cord blood of the infants was strikingly higher than the mean values of the maternal blood. Also, the standard deviation of the infants' blood was considerably higher than that of the mothers' blood.

Table VIII records the paired observations of cord blood and maternal blood. In sixty-eight instances the serum vitamin B_{12} level of the cord blood was greater than that of the mother's blood. (It is recorded as greater if the serum level of the cord blood was more than 50 μμg./ml. greater than the maternal blood, and was regarded as the same if it was no more than 50 μμg./ml. higher than the maternal blood. Even when serum vitamin B_{12} level of cord blood was 1 μμg. less than that of the maternal blood it was recorded as less than the mother's blood.) The values for the cord blood were significantly greater than those of the maternal blood in the majority of observations. Eight of the 110 specimens of cord blood had vitamin B_{12} values of over 1,000 μμg./ml. The scattergram in Figure 2 emphasizes these findings.

There was no correlation of parity or age with the serum vitamin B_{12} values. Similar

TABLE VII

Serum Vitamin B_{12} Levels in Paired Maternal and Cord Blood

Blood	Observation (no.)	Mean (μμg./ml.)	Standard Deviation (μμg./ml.)	Range (μμg./ml.)
Maternal blood obtained within few hours of delivery	32	293	132	<50 to 796
Maternal blood obtained 12 to 72 hours after delivery	133	224	166	<50 to 796
Cord blood of infants	110	496	358	<50 to 2,561

TABLE VIII

Comparison of Vitamin B_{12} Levels in Paired Cord Blood and Maternal Blood

Blood	Observations (no.)
Cord blood level at least 50 μμg./ml. higher than maternal blood	68
Cord blood level equal to or no more than 50 μμg./ml. higher than maternal blood	25
Cord blood level less than maternal blood	12

results were reported by Dumont and Karlin.[18] A dietary history was taken from each patient studied. These studies could give only a semiquantitative estimation of the patient's dietary intake of vitamin B_{12}. Each patient was questioned as to the number of times per week she had eaten beef, pork, veal, fowl, fish and cheese, the number of eggs she had eaten weekly and the amount of milk taken daily. From this information a score was tabulated for each patient. Thus, a point was assigned for every time during the week she had eaten fish, fowl or meat. The scores were then compared with the serum levels and an attempt was made to correlate the vitamin B_{12} levels with these scores. No correlation was found.

It is believed that had the low serum levels been due to a gross dietary deficiency, the large number of patients studied and questioned would have reflected this deficiency. It is possible that lesser degrees of dietary deficiency may not have been reflected by the methods used. The majority of patients studied were from the outpatient department, and came from the lower economic group. Karlin[5] in France, has found that the serum vitamin B_{12} levels of pregnant women from the laboring class are somewhat lower than those of pregnant women from the higher economic brackets.

COMMENTS

A number of possibilities regarding the mechanism of production of low serum levels of vitamin B_{12} in pregnancy and the puerperium may be suggested. Age, parity and gross dietary deficiencies have already been excluded. Diminution of secretion of intrinsic factor, and other mechanisms resulting in an impaired absorption of the vitamin have been excluded by others.[1,19] Because more than 80 per cent of the serum vitamin B_{12} exists in bound form, it is conceivable that the concentration of serum-binding capacity for vitamin B_{12} might be diminished by the hydremia of pregnancy.

We have previously shown[20–22] that the hydremia of pregnancy rapidly disappears in a matter of hours or at most within a few days after delivery. This has been confirmed by others. The much slower curve of regression of the serum vitamin B_{12} level, exhibited postpartum, would exclude antepartum hydremia from playing the predominant role in the low serum levels, compared with those of normal subjects.

Moreover, five normal pregnant women were subjected to comprehensive metabolic, endocrinologic and hematologic studies for ten to fourteen days antepartum and for a like period postpartum.[23] In these women the level of total plasma vitamin B_{12} did not return to that of the normal women as the plasma volume decreased after delivery, again indicating that hydremia of pregnancy alone is not responsible for producing the low serum vitamin B_{12} levels of pregnancy.

It has been shown[20,21,24,30] that maternal erythropoiesis increases during pregnancy and that the total red cell volume increases by 20 to 40 per cent. This would undoubtedly increase the maternal utilization of vitamin B_{12}. The elevated serum values in cord blood as compared with the values of the peripheral blood of normal mothers was first reported by Heinrich[2] and Killander and Vahlquist[25] and has since been confirmed by a number of observers.[8,18,19,26,27] Chow et al.[28] and Hellegers et al.[29] state that a large proportion of absorbed vitamin B_{12} is preferentially taken up by the fetus in both rats and man. Heinrich[2] and Baker et al.[9] found decreased excretion of urinary vitamin B_{12} in pregnancy, and Heinrich believed that human milk was often deficient in vitamin B_{12} content.

These observations have led some to state that the demand of pregnancy, particularly that of the fetus, is high and that the low serum vitamin B_{12} values in pregnancy are due to a deficiency of the vitamin. It has been estimated that the normal person who is not pregnant has tissue stores of 1 to 2 mg. of vitamin B_{12}. Whether the demands of the fetus during pregnancy and increased utilization of this vitamin by the pregnant woman are sufficient to deplete the vitamin stores is not known. Nor is it known whether lowered serum levels without depletion of stores of the vitamin can occur, as for example, when there is increased utilization of the vitamin. One

TABLE IX

Clearance of Injected Vitamin B_{12} from Serum of Normal Pregnant Women and Normal Women ($\mu\mu$g./ml.)

Time	Level	Standard Deviation	Standard Error
Normal Pregnant Women, Third Trimester (Nine)			
Before injection..	148	67	11.6
7 days after	220	97	32.3
15 to 16 days after.	226	92.5	32.8
Normal Women (Eighteen)			
Before injection..	387	175	24
7 days after.....	589	242	57
15 to 16 days after.	474	204	48

NOTE: 150 μg. of vitamin B_{12} was injected intramuscularly. Serum vitamin B_{12} determinations were performed at four hours after the injection and daily thereafter for twenty-three days.

group[29] has suggested that the vitamin is more readily absorbed during pregnancy in human beings and in rats.

Another possible explanation for the lower serum levels would be a decrease during pregnancy of the serum proteins which bind vitamin B_{12}. At present we are studying the possible roles of deficiency of vitamin B_{12} and of decrease of binding capacity. It has been shown by others[32–34] that when vitamin B_{12} is given as an injection, it disappears more slowly from the serum of patients with an increased binding capacity than it does from the serum of persons with normal binding capacity. Twenty-seven women (eighteen normal and nine pregnant) were given 150 μg. of vitamin B_{12} intramuscularly. In this study, comparison of the rate of decrease of vitamin B_{12} in the serum of these two groups from the peak levels showed no significant difference.

However, as shown in Table IX, seven and fifteen days after the injection of 150 μg. of vitamin B_{12} the serum levels of the pregnant women had not returned to the levels before injection but still were significantly lower than the levels of the normal women before injection.

After two control observations, 10 μg. of vitamin B_{12} was given orally each day for three to six months to a group of pregnant women. Vitamin B_{12} levels were followed serially through pregnancy. The mean serum vitamin B_{12} level of this group, before commencing oral administration of the vitamin, was 283.6 $\mu\mu$g./ml. (standard deviation 86.37; standard error 23) and after several months of administration of the vitamin near term the mean level was 222.5 $\mu\mu$g./ml. (standard deviation 88.5; standard error 23.5).

The oral administration of this daily dose of vitamin B_{12} did not result in an increase of serum concentrations or alteration of the normal curve of regression of serum vitamin B_{12} levels during pregnancy.

For some time we have wished to compare *in vitro* and *in vivo* the vitamin B_{12} binding capacity of serum in pregnant women with that in normal women. The literature differs as to whether the capacity of serum to bind vitamin B_{12} is specific or non-specific.

The variable results in the literature may be due to the different methods used[15,16,31,35,36] and to the different increments of vitamin B_{12} added to serum. Most observers have added larger, more widely spaced increments, than we. Increments of cobalt^{58}-vitamin B_{12} ranging from 50 to 6,400 $\mu\mu$g. were added to pooled serum from normal pregnant women and from normal women. Twelve subjects were used for the pooled serum of each group. The initial vitamin B_{12} levels of the pooled serum were determined microbiologically with L. leishmanii. The bound vitamin was determined radiometrically and microbiologically in the supernatant after adsorption with activated charcoal and in the fluid remaining inside the visking casing after ultrafiltration. Free vitamin B_{12} was determined radiometrically and microbiologically in the charcoal after adsorption, and also in the ultrafiltrate.

In Figure 3, using the charcoal methods, it is seen that as increments of Co^{58}-vitamin B_{12} are added to serum, progressive binding of the vitamin occurs up to a maximum level above which the curve flattens, and no further binding takes place within the range of increments

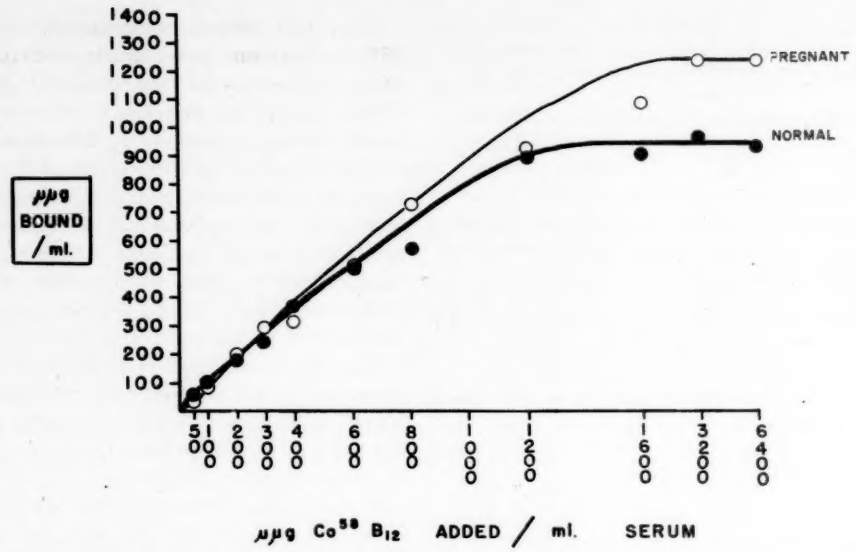

FIG. 3. Vitamin B_{12} binding capacity of pooled serum from normal women and normal pregnant women.

added. (In other experiments it was found that quantities of added Co^{58}-vitamin B_{12} of 9,400 μμg. or more were associated with a further much smaller increase of binding.)

Also it is apparent from this figure that the unsaturated vitamin B_{12} binding capacity of the serum of pregnant women was somewhat greater than the unsaturated binding capacity of the serum of normal women, when measured by the charcoal method.

TABLE X

Incidence of Low Serum Vitamin B_{12} Values in Normal Women, Normal Pregnant Women and in Pregnant Women with Anemia

Subjects	Patients Studied (no.)	Serum Vitamin B_{12} Level (μμg./ml.)		
		150–200	100–149	<100
Normal women...	51	0	1	0
Pregnant women				
First trimester.	34	4	2	0
Second trimester	59	5	2	2
Third trimester.	252	27	15	6
Pregnant women with iron deficiency anemia..	15	2	1	2
Pregnant women with megaloblastic anemia and iron deficiency..	7	1	1	3
Pregnant women without iron deficiency.....	1	0	0	1

The microbiologic assay of vitamin B_{12} of the pooled serum from the pregnant group was 280, as compared with 325 μμg./ml. from the normal group. The normal group were hospital employees of childbearing age. So far the results with ultrafiltration have been similar but have not been identical with those found with the charcoal method; with progressive increments of added vitamin B_{12}, the amount bound shows a rapid rate of increase followed by much slower rate of increase in the ultrafiltration method, similar to the observations of Meyer, Bertcher and Cronkite[37] using the dialysis method.

Table X shows the incidence of low serum vitamin B_{12} values in normal pregnant women and in some patients with anemias of pregnancy. The low serum values in some normal pregnant women were discussed earlier in this paper. Fifteen patients with classic iron deficiency anemia during pregnancy were selected. The criteria used for their selection were hypochromic anemia, decreased iron in bone marrow, low serum iron and high un-

saturated iron-binding capacity, absent stainable iron in the bone marrow, compatible bone marrow changes without megaloblastosis and satisfactory response to iron therapy. Results of folic acid excretion studies were within normal limits in all patients. The serum vitamin B_{12} was 150 to 200 $\mu\mu$g./ml. in two patients, 145 $\mu\mu$g./ml. in another, 28 $\mu\mu$g./ml. in a fourth, 90 $\mu\mu$g./ml. in a fifth patient, and above 200 $\mu\mu$g./ml. in the remainer. Thus, in three of the fifteen patients the serum vitamin B_{12} concentration was significantly reduced and in two of these three patients the levels were well within the range found in patients with pernicious anemia in relapse. A number of patients with normocytic normochromic anemia were similarly studied and showed no abnormality of folic acid excretion or of serum vitamin B_{12} concentration.

Seven patients with combined iron deficiency and megaloblastic anemia of pregnancy were similarly studied. The excretion of folic acid after oral administration was slightly decreased in one patient and was normal after both oral and intramuscular administration in the remainder. Four of seven patients showed serum vitamin B_{12} levels of less than 149 $\mu\mu$g./ml., and in three of these the serum vitamin B_{12} level was less than 100 $\mu\mu$g./ml. Another patient with a hyperchromic macrocytic anemia and an intermediate megaloblastosis of the bone marrow had initial vitamin B_{12} levels of 162 to 174 $\mu\mu$g./ml., which later dropped to less than 50 $\mu\mu$g./ml. This patient showed a satisfactory, although somewhat delayed, response to administration of vitamin B_{12} antepartum, with mild reticulocytosis, rise of hemoglobin, hematocrit and red cells to normal levels for pregnancy, increase of total red cell volume and reversal of the megaloblastosis to normoblastosis. Thus, four of eight patients showed serum vitamin B_{12} levels well within the range which is found in patients with Addisonian pernicious anemia in relapse, and one showed a borderline value. Although the numbers are small it is of interest that the incidence of low serum vitamin B_{12} levels in this group of subjects seems to correspond well with our previously reported response to vitamin B_{12} in four of eight instances of megaloblastic anemia of pregnancy and the puerperium.[1]

It has been found by many observers, including ourselves, that some patients with megaloblastic anemia of pregnancy and the puerperium do not respond to the administration of vitamin B_{12} but do respond to therapy with folic acid and folinic acid. The absence of abnormalities of folic acid excretion in the present group may be due to the relative insensitivity of this test to deficiencies of folic acid. In this connection it is of interest that Baker et al.[9] have stated that in normal pregnancy the concentration of folic acid in the blood and urine is increased (although more recent work by Chanorin et al. suggests that tissue folic acid deficiency may develop in normal pregnant women). Unless a patient with megaloblastic anemia first fails to respond to adequate administration of vitamin B_{12} and then responds to the administration of folic acid, hematologic response to folic acid does not necessarily indicate that the megaloblastic anemia is due to deficiency of folic acid. This has been well established in other types of megaloblastic anemia, such as pernicious anemia. From our data, it seems likely that a deficiency of vitamin B_{12} probably was present in approximately half of the cases we have seen. It is interesting to speculate whether or not in the normal pregnant women showing critically low serum vitamin B_{12} levels and in those with iron deficiency anemias having comparably low serum vitamin B_{12} levels, a megaloblastic anemia of pregnancy would ultimately develop if the length of pregnancy was not limited to approximately nine months.

SUMMARY

The serum vitamin B_{12} level of normal pregnant women is significantly lower than that of normal women. The serum vitamin B_{12} concentration progressively decreases during pregnancy and progressively increases during the early weeks of the postpartum period. The decrease does not seem to be primarily due to the hydremia of pregnancy, or to altered capacity of the serum to bind

vitamin B_{12} during pregnancy, as determined by the charcoal adsorption or ultrafiltration methods for assay of vitamin B_{12} binding capacity.

The serum vitamin B_{12} level of full-term normal infants at birth is considerably greater than that of their mothers.

From the data presented in this paper and from that found in the literature it is not warranted to conclude that a deficiency of vitamin B_{12} develops in normal pregnant women. Further information regarding the state of tissue stores of this vitamin during pregnancy must be obtained before this problem can be settled.

Some patients with megaloblastic anemias of pregnancy and the puerperium observed at the Royal Victoria Hospital have shown serum vitamin B_{12} concentrations comparable to the levels found in patients with addisonian pernicious anemia in relapse and have responded hematologically to administration of the vitamin.

REFERENCES

1. Lowenstein, L., Pick, C. and Philpott, N. Megaloblastic anemia of pregnancy and the puerperium. *Am. J. Obst. & Gynec.*, 70: 1309, 1955.
2. Heinrich, H. C. Biochemical basis of the differential diagnosis and therapy of the vitamin B_{12} deficiencies (B_{12} hypo and avitaminosis) found in man and domestic animals. II. Examination of the B_{12} metabolism in humans during pregnancy and lactation. *Klin. Wchnschr.*, 32: 205, 1954.
3. Lowenstein, L. and Deschenes, E. Serum B_{12} levels and folic acid urinary excretion studies in pregnancy and the puerperium. Proceedings of the Sixth Congress of the International Society of Hematology, Boston, 1956.
4. Karlin, R. Influence of oral or parenteral administration of vitamin B_{12} on the level of this vitamin in human milk. *Bull. Soc. Chim. biol.*, 36: 1665, 1954.
5. Karlin, R. C. R. Vitamin B_{12} and methionine of the blood serum of women during gestation and and lactation. *Compt. rend. Soc. de biol.*, 149: 971, 1955.
6. Boger, W. P., Wright, L. D., Beck, G. D. and Bayne, G. M. Vitamin B_{12}: Correlation of serum concentrations and pregnancy. *Proc. Soc. Exper. Biol. & Med.*, 92: 140, 1956.
7. Boger, W. P., Bayne, G. M., Wright, L. D. and Beck, G. D. Differential serum vitamin B_{12} concentrations in mothers and infants. *New England J. Med.* 256: 1085, 1957.
8. Okuda, K., Kelleger, A. E. and Chow, B. F. Vitamin B_{12} serum level and pregnancy. *Am. J. Clin. Nutrition*, 4: 440, 1956.
9. Baker, H., Erdberg, R., Pashur, I. and Sobotka, H. Study of folic acid and vitamin B_{12} in blood and urine during normal pregnancy. *Proc. Soc. Exper. Biol. & Med.*, 94: 513, 1957.
10. Skeggs, H. R., Mepple, H. H., Valentik, K. A., Huff, A. W. and Wright, L. D. Observations on the use of Lactobacillus leishmanii 4797 in the microbiological assay of vitamin B_{12}. *J. Biol. Chem.*, 5: 184, 1950.
11. Bliss, C. I. Statistics of Bioassay. New York, 1953. Academic Press, Inc.
12. Teply, L. J. and Elvehjem, C. A. Titrimetric determination of "Lactobacillus casei factor" and "folic acid." *J. Biol. Chem.*, 157: 303, 1945.
13. Gregory, M. E. The microbiological assay of vitamin B_{12} in the milk of different species. *Brit. J. Nutrition*, 8: 340, 1954.
14. Gregory, M. E. Some observations on the measurement of binding by intrinsic factor preparations. *Biochem. J.*, 66: 457, 1957.
15. Miller, O. N. Determination of bound vitamin B_{12}. *Arch. Biochem.*, 68: 255, 1957.
16. Miller, O. N. Studies on interaction among serum protein materials containing intrinsic factor and vitamin B_{12}. *Arch. Biochem.*, 72: 8, 1957.
17. Boger, W. P., Wright, L. D., Strickland, S. C., Gylfe, J. S. and Ciminera, J. L. Vitamin B_{12}: correlation of serum concentrations and age. *Proc. Soc. Exper. Biol. & Med.*, 89: 375, 1955.
18. Dumont, M. and Karlin, R. Etude de la teneur en vitamin B_{12} du plancenta du sang maternal et du sang foetal dans l'accouchement. A terme et promature. *Gynec. & Obst.*, 2: 197, 1957.
19. Hallegers, A., Okuda, K., Nesbitt, R. E. L., Smith, D. W. and Chow, B. F. Vitamin B_{12} absorption in pregnancy and in the newborn. *Am. J. Clin. Nutrition*, 5: 327, 1957.
20. Tysoe, F. W. and Lowenstein, L. Blood volume and hematologic studies in pregnancy and the puerperium. *Am. J. Obst. & Gynec.*, 60: 1187, 1950.
21. Lowenstein, L., Pick, C. A. and Philpott, N. W. Correlation of blood loss with blood volume and other hematological studies before, during and after childbirth. *Am. J. Obst. & Gynec.*, 60: 1206, 1950.
22. Philpott, N. W., Pick, C. A. and Lowenstein, L. Blood changes which predispose to shock in the postoperative and post-delivery phase. *New Orleans M. & S. J.*, 103: 559, 1951.
23. Venning, E. H., Dyrenfurth, I., Lowenstein, L. and Beck, J. C. Metabolic studies in pregnancy and the puerperium. In press.
24. Lowenstein, L. and Bramlage, C. A. The bone marrow in pregnancy and the puerperium. *Blood*, 12: 261, 1957.
25. Killander, A. and Vahlquist, B. Vitamin B_{12}

concentration in serum of full-term and premature infants. *Nord. med.*, 51: 777, 1954.

26. Boger, W. P., Bayne, G. M., Wright, L. D. and Beck, G. D. Differential serum B_{12} concentrations in mothers and infants. *New England J. Med.* 256: 1085, 1957.
27. Dixit, G. H., Mody, B. N., Jhala, H. I., Parekh, J. G. and Ramasarma, G. B. A note on serum vitamin B_{12} concentrations in parturient mothers and the cord blood of their infants. *Indian J. M. Sc.*, 11: 145, 1957.
28. Chow, B. F., Barrows L. and Ling, C. T. The distribution of radioactivity in the organs of the fetus or of young rats born by mothers injected with vitamin B_{12} containing Co^{60}. *Arch. Biochem.*, 34: 151, 1951.
29. Hellegers, A., Okuda, K., Nesbitt, R. E. L., Smith, D. W. and Chow, B. F. Vitamin B_{12} absorption in pregnancy and in the newborn. *Am. J. Clin. Nutrition*, 5: 327, 1957.
30. Katon, W. L., Robbie, C. C., Reid, D. E., Caswell, R., Maletkos, C. J., Fhuharty, R. G. and Gibson, J. G. The circulating red cell volume and body hematocrit in normal pregnancy and the puerperium by direct measurement using radioactive red cells. *Am. J. Obst. & Gynec.*, 61: 1207, 1951.
31. Ross, G. I. M. Vitamin B_{12} assay in body fluids using euglena gracilis. *J. Clin. Path.*, 5: 250, 1952.
32. Mollin, D. L., Pitney, W. R., Baker, S. J. and Bradley, J. E. The plasma and urinary clearance of parenterally administered Co^{58} B_{12}. *Blood*, 7: 31, 1956.
33. Miller, A., Corbins, H. F. and Sullivan, J. F. The plasma disappearance excretion and tissue distribution of B_{12} in normal and leukemic subjects. *J. Clin. Invest.*, 36: 18, 1957.
34. Jones, P. N., Mills, E. H. and Copps, R. B. The effect of liver disease on serum B_{12} concentrations. *J. Lab. & Clin. Med.*, 49: 910, 1957.
35. Pitney, W. R., Beard, M. F. and Van Loon, E. V. Observations on the bound form of vitamin B_{12} in serum. *J. Biol. Chem.*, 207: 134, 1954.
36. Bertcher, R. W. and Meyer, L. M. Co^{60} vitamin B_{12} binding capacity of normal human serum. *Proc. Soc. Exper. Biol. & Med.*, 94: 169, 1957.
37. Meyer, L. M., Bertcher, R. W. and Cronkite, K. P. Serum Co^{60} Vitamin B_{12} binding capacity in some hematologic disorders. *Proc. Soc. Exper. Biol. & Med.*, 96: 360, 1957.

Radiation Comparison of Cobalt Isotopes

CHARLES ROSENBLUM, PH.D.

THE VALUE of radioactive vitamin B_{12}[1–5] has been established as a diagnostic agent in pernicious anemia and related diseases, as a tracer for this vitamin in animal and *in vitro* studies, and as an analytic agent for certain types of isotope dilution procedures and biologic potency determinations. The radioactive constituent is a cobalt isotope, the properties of which determine the sensitivity of detection of the vitamin and the radiation dose which a test subject receives.

HALF-LIFE

Factors which influence the choice of radioactive isotope are (1) half-life (T), (2) availability, (3) specific activity (radioactivity per unit weight), and (4) the nature and energies of radiations emitted. Of about twelve radioactive cobalt isotopes reported,[6] all but four are eliminated because their rates of radioactive decay are inconveniently short, i.e., reported half-lives range between 0.2 second and 18 hours. The four remaining isotopes, those with atomic mass numbers 56, 57, 58 and 60 and respective half-lives of 77 days, 270 days, 72 days and 5.27 years, have all been successfully employed for the labeling of vitamin B_{12}. Their decay rates are sufficiently slow to yield useful products, despite the time required for microbiologic synthesis, isolation and purification of the labeled vitamin.

AVAILABILITY

Cobalt^{60} has been used most frequently because it is in constant supply and readily obtainable from the U. S. Atomic Energy Commission[7] and comparable regulatory agencies in other countries producing and distributing radioisotopes. It is produced in a nuclear fission reactor as a result of neutron (n) capture by stable cobalt (mass number 59), with simultaneous emission of gamma radiation. This (n, γ) reaction is the most common type of neutron reaction and may be represented as $Co^{59}(n, \gamma)\ Co^{60}$.

Cobalt^{58} is also supplied by the U. S. Atomic Energy Commission.[7] It is also prepared in a fission reactor, but from separated isotope nickel^{58} by neutron capture with emission of a proton (p), i.e., by the reaction $Ni^{58}(n, p)\ Co^{58}$. Unfortunately, a trace of nickel^{60} present in the nickel target gives rise to contamination by cobalt^{60} which, although unimportant initially, becomes more evident as the cobalt^{58} decays. A trace of iron isotope Fe^{59} is also present in cobalt^{58}.

In contrast to the fission reactor source of the preceding isotopes, cobalt^{56} and cobalt^{57} are prepared in an ion accelerator such as the cyclotron. Cobalt^{56} is produced by bombarding iron targets with deuterons (d) or protons which lead to mixtures of cobalt^{56} and cobalt^{57} by nuclear reactions such as $Fe^{56}(d, 2n)\ Co^{56}$ and $Fe^{56}(d, n)\ Co^{57}$. Similarly, cobalt^{57} may be formed by interaction of a proton beam with highly purified nickel (free of cobalt and iron contamination) in which the principal reaction induced is $Ni^{58}(p, pn)\ Ni^{57}$ followed by rapid decay of nickel^{57} to cobalt^{57}. Although cobalt^{56} has been available in limited quantities for some time, supplies of cobalt^{57} have only recently become assured.[8]

SPECIFIC ACTIVITY

Detection sensitivity is determined primarily by the specific activity of the radioactive substance. The specific activity of radioactive vitamin B_{12} is determined by the specific activity of the cobalt present initially in the

From Merck Sharp & Dohme Research Laboratories, Division of Merck & Co., Inc., Rahway, New Jersey.

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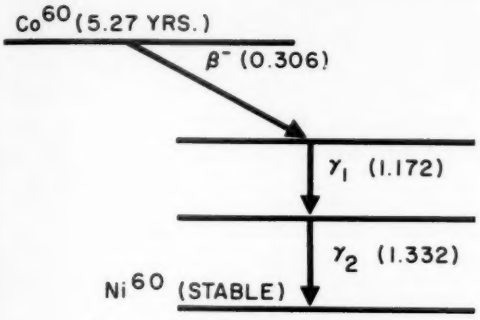

FIG. 1. Decay scheme for cobalt[60].

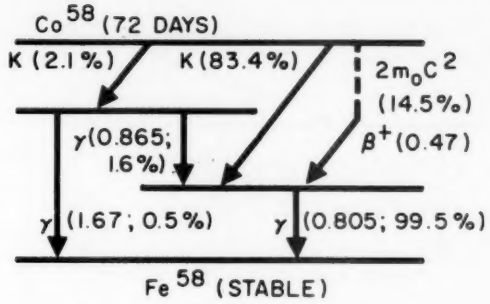

FIG. 2. Decay scheme for cobalt[58].

medium in which microbiologic synthesis of the vitamin occurs. Although metallic cobalt sources are offered by the U. S. Atomic Energy Commission[7] with activities up to 50 mc./mg. Co, the most active cobalt[60] received by Merck & Co., Inc., in the form of soluble cobalt salt, has had at best a specific activity of ≃20 to 25 mc./mg. Accordingly the highest vitamin B_{12}-Co^{60} activity attainable is ≃1 mc./mg. since the cobalt content of the vitamin is 4.34 per cent. This is the specific activity of the radioactive vitamin marketed and can be exceeded only if cobalt[60] of higher activity is provided. In such a preparation, approximately 1 cobalt atom per 50 is radioactive (i.e., Co^{60}).

No such limitation attaches to labeling of vitamin B_{12} with Co^{58}. This isotope is supplied in "carrier-free," essentially isotopically pure form, and must be diluted only to the extent that exogenous cobalt is required by a particular strain of microorganism to produce a suitable yield of vitamin. It has been shown,[9] for example, that a strain of Streptomyces griseus produces a maximum yield in a nutrient medium containing 1 to 2 p.p.m. cobalt, although significant yields of vitamin have been produced at cobalt concentrations of 0.05 to 0.1 p.p.m. Since cobalt[58] is essentially weightless, even in quantities of 20 mc. (equivalent to 0.64 μg. Co), stable cobalt (cobalt[59]) must be added to a medium to insure a reasonable yield. This balancing of size batch and yield with resultant specific activity led Merck & Co., Inc., to offer a product of 5 mc./mg. vitamin B_{12}.

Similar considerations would apply to cobalt[56] and cobalt[57]. Vitamin labeled with the former isotope is available from Glaxo Laboratories in London. Specific activities[5,10] of 12 mc./mg. of vitamin B_{12}-Co^{56} and 13.5 mc./mg. of vitamin B_{12}-Co^{57} have been reported. In 1950 we produced a $Co^{56,57}$-labeled preparation for use in human subjects by Dr. Marshall Brucer, Oak Ridge Institute of Nuclear Studies. Cobalt[57] of high specific activity and isotopic purity has not been available in quantity until recently.[8] It would be desirable to have a source of vitamin B_{12}-Co^{57}, with an activity of ≃5 mc./mg. for use by investigators. With cobalt[57] available, this becomes a distinct possibility. The superiority of cobalt[57] over cobalt[58] in terms of useful life-time is evident.

In cobalt-labeled vitamin with a specific activity of 5 mc./mg., there is present approximately 1 cobalt[56] or 1 cobalt[58] atom per 300 total cobalt atoms, while in the form labeled with cobalt[57], the ratio is 1 per 85.

DECAY SCHEMES

The types, energies and sequence of emission of radiation by a radioactive nucleus are depicted diagrammatically in what is called a decay scheme. Figures 1 through 4 are the accepted diagrams for the four isotopes in question. Exact values of branching or energies may be subject to some revision, but the following considerations will not be effected significantly. Cobalt[60] is seen (Fig. 1) to emit in cascade one beta (β^-) ray (0.306 Mev) and two gamma (γ) rays (1.172 and 1.332 Mev) per disintegration.[11] Cobalt[58] is seen (Fig. 2) to undergo branching decay,[12,13] i.e., 14.5 per cent of disintegrations proceed by positron (β^+)

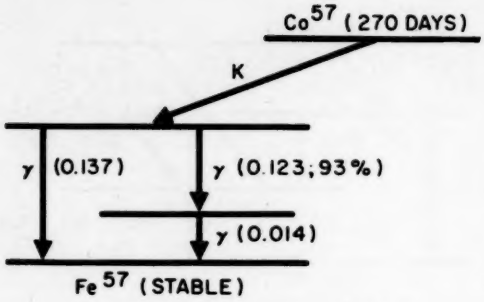

FIG. 3. Decay scheme for cobalt[57].

emission (0.47 Mev) while 85.5 per cent are accompanied by electron capture (K). Both modes of decay are followed by emission of gamma rays (0.805 Mev, 99.5 per cent; 0.865 Mev, 1.6 per cent; 1.67 Mev, 0.5 per cent). In addition, annihilation of each positron produces two 0.51 Mev gamma rays. Cobalt[57] (Fig. 3) decays[14] entirely by electron capture, followed by branched emission of gamma rays of 0.137 Mev ($\simeq$7 per cent of disintegrations) and by a sequence of 0.123 and 0.014 Mev rays (93 per cent). Cobalt[56] has the most complicated decay pattern[13,15,16] (Fig. 4), emitting eleven gamma rays plus annihilation radiation following 19.2 per cent decay by positron (1.47 Mev) emission and the remainder by a complex combination of electron capture processes. The eleven different gamma rays emitted, exclusive of annihilation (two 0.51 Mev rays per β^+ emission) are listed in Table I, which also shows the number (in per cent columns) of the several gamma rays produced per 100 disintegrations.

TABLE I
Emission of Gamma Rays by Co[56]

Mev	Per Cent	Mev	Per Cent
3.47	1.1	1.35	5.7
3.25	11.5	1.22	69.6
2.98	1.9	1.025	15.6
2.56	15.4	0.975	1.8
2.02	11.4	0.845	98.9
1.76	16.7	—	—

DETECTION SENSITIVITY

With the exception of cobalt[57], each of these isotopes can be detected by conventional Geiger tubes sensitive to particulate radiation. Of these isotopes, cobalt[60] is most readily detected since each disintegration is accompanied by ejection of a beta particle. Despite the emission by cobalt[58] and cobalt[56] of positrons of greater energy than that of the cobalt[60] beta particle, positron emission accounts for only a fraction (14.5 and 19.2 per cent) of the branching; and, for equal radioactivities, beta counting is a more sensitive procedure for cobalt[60] than for the other two nuclides. This would apply to liquid scintillation methods as well as to gas discharge tubes.

This superiority is not maintained in the solid scintillation counting device commonly employed in clinical laboratories. Gamma radiation (as in cobalt[60]) of high energy penetrates matter more readily but is less readily absorbed by a phosphor such as NaI(Tl) than is less energetic gamma radiation as is emitted by cobalt[58] or cobalt[57]. Thus the greater attenuation of gamma radiation from the latter isotopes, as compared to that from cobalt[60] and cobalt[56], during the passage of radiation through a given medium (i.e., tissue or sample) is compensated in part by greater absorption in the phosphor crystal. Furthermore, the thallium-activated NaI scintillation crystal has a maximum scintillation efficiency[17] at $\simeq$0.1–0.15 Mev which is extremely favorable to cobalt[57]. One would expect that, in the absence of a great depth of absorbing medium and where thin NaI(Tl) crystals are employed, gamma scintillation detection efficiency would be greatest for cobalt[57] and least for cobalt[60], with cobalt[58] probably intermediate. An accurate intercomparison has not yet been reported and results would depend on the conditions of measurement.

MAXIMUM PERMISSIBLE CONCENTRATIONS

Safety requirements impose limits on the amounts of a radioisotope which may be administered to a human subject. Maximum permissible body concentrations (MPC) are computed on the basis of the known distribution of isotope in critical organs and an accepted permissible whole body radiation dose rate such that blood-forming organs receive no

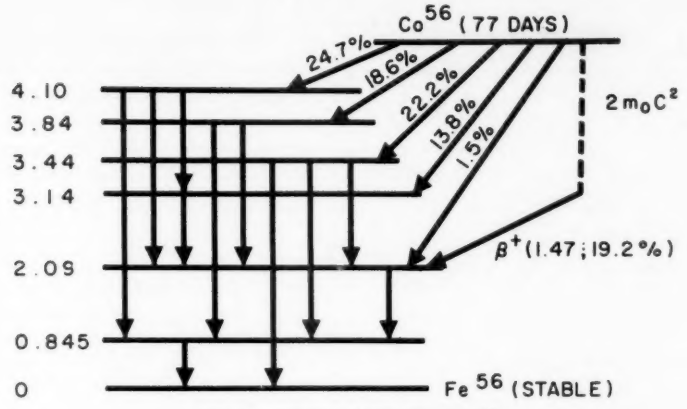

FIG. 4. Decay scheme for cobalt[56].

more than 300 milliroentgens (mr.) per week. This official limit is set by the U. S. Atomic Energy Commission for occupational exposure,[18] and is based upon figures published in its Handbook 52 by the National Bureau of Standards.[19] Although revisions of these limits have been recommended by the National Committee on Radiation Protection and Measurements, these have not been officially adopted as of this symposium date; and all maximum permissible tissue concentrations will be computed upon the current basis of 300 mr./week.

According to Handbook 52, the maximum permissible total number of microcuries of a given isotope which will result in a dose rate* of 300 rep/week is

$$q = \frac{2.6 \times 10^{-3} \, m \, W}{\Sigma \, (b \, E) \, f_2}$$

where m is the mass (1,700 gm. for liver) of the critical organ, Σ (b E) is the effective (absorbed) energy of radiation (of E in Mev units) per disintegration, and f_2 is the fraction of total body isotope which is present in the target organ. Absorption coefficients of beta rays, positrons and gamma rays are included in computations of b as described in the handbook. The effective liver diameter is taken as 10 cm. If W is 0.3 rep/week adopted as maximum permissible radiation dose rate, q becomes equal to the MPC in microcuries. Computed values of Σ(b E) and q for the four cobalt isotopes in question are listed in Table II on the assumption that we are dealing with cobalt concentrated in the liver as the target organ with an f_2 of 0.68. These MPC's (q) represent the number of microcuries of cobalt isotope which will deliver radiation to the target organ at a dose rate of 300 rep/week. Obviously, the isotope tolerable in largest amount (of radioactivity) is cobalt[57], (MPC of 40 μc. compared to the small MPC value of $\simeq$2 μc. for cobalt[56] and $\simeq$3 μc. for cobalt[60]).

This in itself is not, however, the sole criterion of usefulness, since these are instantaneous values and do not take into account the decay rates of these isotopes which determine the total absorbed radiation energy, and the frequency with which repeated doses may be administered. Column 4 of Table II contains values of total rep delivered to the liver during complete decay of the MPC's (in the absence of biological elimination or turnover). These integrated doses are equal to (0.3 $\times$ T)/0.693, where T is the effective half-life, here taken as the radioactivity half-life in weeks. The actual total exposure should take into account the biologic half-life (Tb) of the element. This is given as 8.4 days for cobalt in the liver. Accordingly, the total doses delivered to the

* The roentgen (r) applies strictly only to x- and gamma rays. For isotopes which emit particulate radiation, the term "roentgen equivalent physical" (rep) applies. In this discussion, no distinction is made between r and rep since all radiations emitted by the cobalt isotopes are considered to have the same relative biologic effectiveness.

TABLE II
MPC and Cumulative Exposures Due to Radioactive Cobalt Isotopes

Isotope	Σ(b E)	MPC (μc.)	Total rep to Liver Containing MPC				
			Tb Infinite	Tb = 600 Days	Tb = 400 Days	Tb = 180 Days	Tb = 8.4 Days
Co^{56}	0.963	2.0	4.8	4.2	4.0	3.3	0.47
Co^{57}	0.0488	40.0	16.7	11.5	10.0	6.7	0.50
Co^{58}	0.293	6.7	4.5	4.0	3.8	3.2	0.46
Co^{60}	0.736	2.7	117.0	28.0	20.0	10.0	0.52

liver during the effective half-life* of the isotope, if present as elemental cobalt, would be those compiled in the last column of Table II. Between this and the column (fourth) representing absence of elimination are listed cumulative rep values for Tb = 180, 400 and 600 days, which pertain more properly to cobalt administered in the form of vitamin B_{12}.

It is clear that the most favorable (largest) MPC is 40 μc. of Co^{57} and the smallest is 2 to 3 μc. for Co^{56} and Co^{60}. This is primarily a question of type of radiation, or differences in Σ bE values and, in the case of Co^{57}, reflects in part the absence of particulate radiation. Cobalt^{57} thus becomes the preferred cobalt isotope from the point of view of radiation safety,† even when a q comparison is made of permissible microcurie burdens for equal cumulative doses. Because of the difference in decay rates, the cumulative dose delivered to tissue is potentially greatest for cobalt^{60}, which is thus the least desirable of the isotopes for use in human subjects. For cobalt salts, however, with a biologic half-life of 8.4 days, rapid turnover equalizes the radiation dosage delivered by MPC's of the several isotopes, although the values of MPC remain unchanged.

CASE OF RADIOACTIVE VITAMIN B_{12}

For longer terms of residence in the body as is known to be the case with vitamin B_{12}, cumulative doses would be intermediate between those for cobalt and those for zero elimination (Table II). Biologic half-lives ranging as high as two years[20,21] have been reported. Although these values of Tb influence relative total radiation exposures, they do not determine the MPC for a labeled vitamin. The maximum permissible concentrations of the several labeled modifications are not known, primarily because the value of f_2 in the human body is unknown for vitamin B_{12}. One may expect the relative MPC's for vitamin B_{12} labeled with the several isotopes to be the same as for elemental cobalt isotopes, since the liver is a target organ. For want of this knowledge for the several modes of administration, and lacking information as to how f_2 varies with dosage level, the administration of vitamin B_{12}-Co^{60} to human subjects has been limited to the 3 μc. dose set for elemental cobalt^{60}. In all probability f_2 for vitamin B_{12} is less than 0.68. In one study of a human subject[22] an injection of $Co^{56,57}$-labeled vitamin B_{12} had been given to a patient who died. At autopsy two days later the liver f_2 had a value of $\lesssim$0.3 which would suggest that total body MPC for various isotopic modifications of vitamin B_{12} would be severalfold higher than the values computed for Table II. On the other hand, a value of 0.65 six months after an injection of a 3 μg. dose of vitamin B_{12}-Co^{60} has been reported.[23] Lower fractional liver retention is found in animals; 0.1 to 0.4 in the mouse, hamster and rat.[24,25] The 3 μc. limit on vitamin B_{12} has been applied to the oral administration of vitamin B_{12}-Co^{60}, despite the knowledge that only a fraction of the oral dose is actually absorbed.[26,27] This limit adopted for oral

* Effective half-life is defined[19] as $T = \frac{Tb \times Tr}{Tb + Tr}$ where Tb is the biologic half-life of the element and Tr the radioactivity half-life of the isotope. When the turnover is slow or negligible, as occurs with prolonged retention, $Tb \gg Tr$ and effective half-life is equal to Tr.

† This advantage applies also to handling during synthesis of the vitamin, shipment and storage, since shielding requirements are much less rigorous.

cobalt^{60}-labeled vitamin B_{12}, and the proportionately higher MPC's calculable for the other isotopes, is low by a factor which would depend on the amounts administered. For example, oral absorption of vitamin B_{12} is only $\simeq$40 per cent of a 2 μg. dose and $\simeq$20 per cent of a 5 μg. dose, so that MPC's should be raised accordingly. Currently, however, the MPC's of Table II should be valid although based upon a cobalt instead of a vitamin B_{12} pattern. When sufficient information concerning the distribution of ingested vitamin B_{12} in organs of the human body becomes available, revision of MPC values may be practical.

ADDENDUM

Since delivery of this paper, the National Committee on Radiation Protection has recommended certain changes in exposure limits which have the effect of lowering the permissible exposure to certain parts of the body. These recommendations have been incorporated in Handbook 69 which will replace the current Handbook 52. The U. S. Atomic Energy Commission is also contemplating adoption of these regulations for Commission personnel, contractors and licensees as has been announced in the Federal Register of May 2, 1959. This announcement proposed a new table of maximum body burdens of radioisotopes which now include cobalt^{57} and cobalt^{58}. Total body burdens for cobalt isotopes are, however, listed as 10 μc. for cobalt^{60}, 30 μc. for cobalt^{58} and 200 μc. for cobalt^{57}. This constitutes an increase over MPC's of Handbook 52 of three- to fivefold.

REFERENCES

1. Chaiet, L., Rosenblum, C. and Woodbury, D. T. Biosynthesis of radioactive vitamin B_{12} containing Cobalt-60. *Science*, 3: 601, 1950.
2. Rosenblum, C. and Woodbury, D. T. Cobalt-60 labeled vitamin B_{12} of high specific activity. *Science*, 113: 215, 1951.
3. Smith, E. L., Hockenhull, D. J. D. and Quilter, A. R. J. Tracer studies with B_{12} vitamins. 2. Biosynthesis of vitamin B_{12} labelled with ^{60}Co and ^{32}P. *Biochem. J.*, 52: 387, 1952.
4. Bradley, J. E., Smith, E. L., Baker, S. J. and Mollin, D. L. The use of the radioactive isotope of Cobalt Co^{58} for the preparation of labelled vitamin B_{12}. *Lancet*, 476: 477, 1954.
5. Mollin, D. L. and Smith, E. L. The absorption of vitamin B_{12} and the pathogenesis of vitamin B_{12} deficiency. Proceedings of the International Conference on Peaceful Uses of Atomic Energy, Volume 10, page 475. New York, 1956. United Nations.
6. Strominger, D., Hollander, J. M. and Seaborg, G. T. Table of isotopes. *Rev. Modern Physics*, 30: 585 (1958).
7. U. S. Atomic Energy Commission, Oak Ridge National Laboratory: Radioisotopes, Special Materials and Services (Catalogue and Price List), pp. 80, 81, 154. January, 1957.
8. Gleason, G. I. Abbott Radiopharmaceutical Division, Oak Ridge, Tennessee. Personal communication.
9. Hendlin, D. and Ruger, M. L. The effect of cobalt on the microbial synthesis of LLD active substances. *Science*, 111: 541, 1950.
10. Booth, C. C. and Mollin, D. L. Plasma, tissue and urinary radioactivity after oral administration of ^{56}Co-labelled vitamin B_{12}. *Brit. J. Haemat.*, 2: 223, 1956.
11. Way, K., King, R. W., McGinnis, C. L. and van-Lieshout, R. Nuclear Level Schemes, U. S. Atomic Energy Commission TID-5300, p. 70. Washington, D. C., June 1955.
12. Frauenfelder, H., Levine, N., Rossi, A. and Singer, S. Decay of Co^{58}. *Physical Rev.*, 103: 352, 1956.
13. Cook, C. S. and Tomnovec, F. M. Gamma radiation from Co^{56} and Co^{58}. *Physical Rev.*, 104: 1407, 1956.
14. Booth, C. C. and Mollin, D. L. Plasma, tissue and urinary radioactivity after oral administration of ^{56}Co-labelled vitamin B_{12}. *Brit. J. Haemat.*, 2: 58, 1956.
15. Sakai, M. Angular correlation of gamma rays from Fe^{56}. *J. Phys. Soc. Japan*, 10: 729, 1955.
16. Poppema, O. J., Siekman, J. G., vanWageningen, R. and Tolhoek, H. A. *Physica*, 21: 223, 1955.
17. Bell, P. R. In Siegebahn, K., Beta- and Gamma-Ray Spectroscopy, p. 154. New York, 1955. Interscience Publishers, Inc.
18. U. S. Atomic Energy Commission: Title 10—Atomic Energy, Chapt. 1. Atomic Energy Commission, Part 20. Standards for Protection Against Radiation. *Federal Register*, 22: 548, 1957.
19. U. S. Department of Commerce, National Bureau of Standards, Handbook 52: Maximum Permissible Amounts of Radioisotopes in the Human Body and Maximum Permissible Concentrations in Air and Water. Washington, D. C., March 20, 1953.
20. Schloesser, L. L., Deshpande, P. and Schilling, R. F. Biological turnover of cyanocobalamin (vitamin B_{12}) in human liver. *Arch. Int. Med.*, 101: 306, 1958.
21. Glass, G. B. J. Radioactive vitamin B_{12} in the

liver. III. Hepatic storage and discharge of Co^{60} B_{12} in pernicious anemia. *J. Lab. & Clin. Med.*, 52: 875, 1958.

22. BRUCER, M. Oak Ridge Institute of Nuclear Studies. Personal communication.
23. CRONKITE, E. P., HENLEY, E., DRISCOLL, D. H., MEYER, L. M., DOHAN, F. C., RUBINI, J. R. and WOLINS, W. Studies on the kinetics of intravenously injected ^{60}Cobalt labeled vitamin B_{12} in man. Proceedings of the Seventh International Congress of Hematology, Rome, 1958.
24. ROSENBLUM, C., CHOW, B. F., CONDON, G. P. and YAMAMOTO, R. S. Oral versus parenteral administration of Co^{60}-labeled vitamin B_{12} to rats. *J. Biol. Chem.*, 198: 915, 1952.
25. MILLER, A., GAULL, G., ROSS, J. F. and LEMON, H. M. Tissue distribution of parenteral Co^{60} vitamin B_{12} in mouse, hamster, rat and guinea pig. *Proc. Soc. Exper. Biol. & Med.*, 93: 33, 1956.
26. GLASS, G. B. J., BOYD, L. J. and STEPHANSON, L. Intestinal absorption of vitamin B_{12} in humans as studied by isotope technic. *Proc. Soc. Exper. Biol. & Med.*, 86: 522, 1954.
27. SWENSEID, M. E., GASSTER, M. and HALSTED, J. A. Limits of absorption of orally administered vitamin B_{12}: Effect of intrinsic factor sources. *Proc. Soc. Exper. Biol. & Med.*, 86: 834, 1954.

Distribution of Vitamin B_{12} Between Plasma and Cells

HARRY SOBOTKA, PH.D.,* HERMAN BAKER, PH.D† AND HERMAN ZIFFER, M.D.‡

THE analytical values for vitamin B_{12} are nearly always given for serum or plasma. Since vitamin B_{12} assays may be performed on whole blood without inhibition of the assay micro-organism and without interference by color, we investigated the distribution of vitamin B_{12} between plasma and the cellular elements of the blood. A preliminary report of these findings has been published.[1]

We studied blood from (1) three normal healthy control subjects, (2) four recently diagnosed, still untreated patients with pernicious anemia and (3) a patient with total gastrectomy, who had been given vitamin B_{12} orally, but whose serum level was still very low.

METHOD

The blood was citrated or heparinized, the blood cells were separated by centrifugation and washed six times with acid citrate glucose solution. They were then suspended in a volume of this solution equal to the original volume of whole blood. The microbiologic assay was carried out using Euglena gracilis and Ochromonas malhamensis as described by us on previous occasions.[2] Table I gives the values obtained with euglena, which showed excellent agreement with the ochromonas values.

The figures given in Table I show the vitamin B_{12} content for 1 ml. of plasma (p) and the vitamin B_{12} content for 1 ml. of cells (c). The content for the whole blood, given in the next column, is thus

$$t = \frac{c.H + p(100 - H)}{100}$$

where H is the hematocrit reading.

RESULTS

The determination on three normal subjects shows that their plasma contained two to four

TABLE I
Vitamin B_{12} Content of Plasma, Red Blood Cells and Whole Blood

Subject	Vitamin B_{12} ($\mu\mu$g./ml.)			Hematocrit (%)
	Plasma	Red Blood Cells	Whole Blood	
Normal Patients				
1	620	170	425	44
2	600	140	410	42
3	320	185	260	47
Patients with Pernicious Anemia				
1	32	115	45	15
2	23	95	35	15
3	76	30	67	20
4	34	55	38	20
Gastrectomized Patient				
1	50	165	75	20
Average of abnormal cases	43	92	52	—

From the Department of Chemistry, The Mount Sinai Hospital, New York, New York.

* Director; † Research Associate; ‡ Research Assistant.

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times as much vitamin B_{12} as the cells, volume per volume. In the patients with pernicious anemia (and in the gastrectomized patient) the situation is reversed; the cellular vitamin B_{12} level is, on the average, about 90 $\mu\mu$g. as compared with 165 $\mu\mu$g. in the normal subjects. The plasma level dropped from an average of 500 $\mu\mu$g. in normal subjects to about one-twelfth this value; namely 40 $\mu\mu$g. in the patients with pernicious anemia. With the exception of Case 3 (pernicious anemia) there is actually more vitamin B_{12} in 1 ml. of cells than in 1 ml. of plasma.

In order to learn the ratio between plasma and cells in 1 ml. of whole blood, it is necessary to multiply the figures given in Table I, "c" with the hematocrit reading and "p" with (100 − hematocrit), respectively. Accordingly, in normal serum the cells carry, in general, not more than one-fifth to one-sixth of the total vitamin B_{12}; in pernicious anemia their share is between one-third and almost one-half.

COMMENTS

It is surprising that cells survive at such low vitamin B_{12} levels in the plasma, even when it is considered that their number is substantially reduced. The relatively high proportion of vitamin B_{12} in the cells of the patients with pernicious anemia suggests that vitamin B_{12} used for red cell formation and maturation is efficiently drawn upon, even when the pool of the vitamin is severely depleted. The abnormal cytologic picture in pernicious anemia may be explained by there being enough vitamin B_{12} to initiate cell formation, but not enough for the cells to mature normally. Only plasma well supplied with vitamin B_{12} supports the normal maturation process, in other words the two systems are not independent from each other.

In pernicious anemia, the bone marrow competes with other tissues for the limited store of vitamin B_{12}. When nerve tissue becomes depleted of the vitamin, subacute combined degeneration of the spinal cord ensues. Treatment of pernicious anemia with folic acid may induce both a hematologic remission and subacute combined degeneration of the spinal cord. This suggests that the small store of vitamin B_{12} is mobilized by the action of folic acid towards the bone marrow, alleviating its deficiency, but markedly aggravating vitamin B_{12} depletion in other tissues.

We do not wish at this time to speculate about the mechanism by which this is accomplished; evidently, the cells contain a strong binding factor. Considering this observation from the teleologic point of view, it serves as a reminder of the conservation of protein during starvation in the most vital organs, brain and heart, while muscles and viscera become depleted, or, in a more closely related field the retention of vitamin B_{12} in the fetus in preference to the maternal organism.[3,4]

SUMMARY

Plasma levels reflect closely the vitamin B_{12} supply of the tissues, even though the blood cells from patients with pernicious anemia often contain more than half as much vitamin B_{12} as do normal cells.

REFERENCES

1. Baker, H., Pasher, I., Sobotka, H., Hutner, S. H., Aaronson, S. and Ziffer, H. Vitamin B_{12} distribution between plasma and cells. *Nature*, London, 180: 1043, 1957.
2. Baker, H., Sobotka, H., Pasher, I. and Hutner, S. H. Comparative study of vitamin B_{12} assay in urine. *Proc. Soc. Exper. Biol. & Med.*, 91: 636, 1956.
3. Baker, H., Erdberg, R., Pasher, O. and Sobotka, H. A study of folic acid and vitamin B_{12} in blood and urine during normal pregnancy. *Proc. Soc. Exper. Biol. & Med.*, 94: 513, 1957.
4. Baker, H., Ziffer, H., Pasher, I. and Sobotka, H. A comparison of maternal and fetal folic acid and vitamin B_{12} at parturition. *Brit. M. J.*, 1: 978, 1958.

Metabolic Turnover of Vitamin B_{12} in the Normal and Diseased Liver

GEORGE B. JERZY GLASS, M.D.* AND WALTER L. MERSHEIMER, M.D.†

IT HAS been known for a long time that the liver is the main storage organ for vitamin B_{12}.[1-4] The mechanisms governing the metabolic turnover of vitamin B_{12} are not yet fully understood, however. Radioactive technics have now made it possible to study them in more detail. This paper deals with some of the data we have collected by means of isotope technics on the metabolic turnover of vitamin B_{12} in the normal and diseased liver.

MEASUREMENT OF HEPATIC UPTAKE OF RADIOACTIVE VITAMIN B_{12}

Several years ago, we developed a technic for counting radioactive vitamin B_{12} in visceral organs *in vivo*,[5] a technic based on the same principle as that involved in counting radioactive iodine in the thyroid.

In accordance with a recent modification of this method,[6] a tracer dose of 0.5 μg. of Co^{60}-vitamin B_{12} or Co^{58}-vitamin B_{12}, containing 0.4 to 0.5 μc. of radioactive cobalt, is given orally in the morning. Twenty-four hours later, 1 ounce of castor oil is administered and, the next morning, a cleansing enema is given. Forty-eight hours after administration of the tracer, counts are taken over the anterior and lateral projections of the liver, over two abdominal control areas, below and to the left of the umbilicus and over the posterior midarea of the left gastrocnemius, which serves as body background. Counts are taken for five minutes each, with the scintillation counter in direct contact with the skin. After deduction of the body background, the hepatic uptake is then calculated in counts per minute and per 1 μc. of radioactive cobalt.

Following oral administration of radioactive vitamin B_{12}, the abdominal counts are initially higher than those over the liver. If a cathartic and enema are given on the third day, however, the abdominal radioactivity decreases below that of the liver, making it possible for the hepatic uptake of vitamin B_{12} to be measured as early as forty-eight hours after administration of the tracer.

In normal subjects, and in patients with simple hypo- or anacidity, the oral administration of radioactive vitamin B_{12}, alone or with intrinsic factor, results in an accumulation of radioactivity over the liver, as shown in Figure 1. With our technic, this amounts to from 400 to 1,200 counts per minute for each microcurie of Co^{60}-vitamin B_{12} ingested.

In pernicious anemia[5,6] or following total gastrectomy,[7] when intrinsic factor is absent, the absorption of vitamin B_{12} from the intestine is virtually abolished, and the hepatic uptake is either zero or present only in traces, as shown by the light squares in Figure 1. When an intrinsic factor preparation is added, intestinal absorption and hepatic uptake become normal, as shown by the black squares.

In most patients with sprue, vitamin B_{12} is not absorbed in the intestine and the hepatic uptake is either zero or present only in traces.[8-10] In contrast to its action in patients with pernicious anemia, however, intrinsic factor administration in patients with sprue does *not* improve the hepatic deposition of vitamin B_{12},[8] because this defect is due, not to the absence of intrinsic factor, but to a

From the Departments of Medicine and Surgery, New York Medical College, New York, New York.

* Associate Professor of Medicine; † Associate Professor of Surgery.

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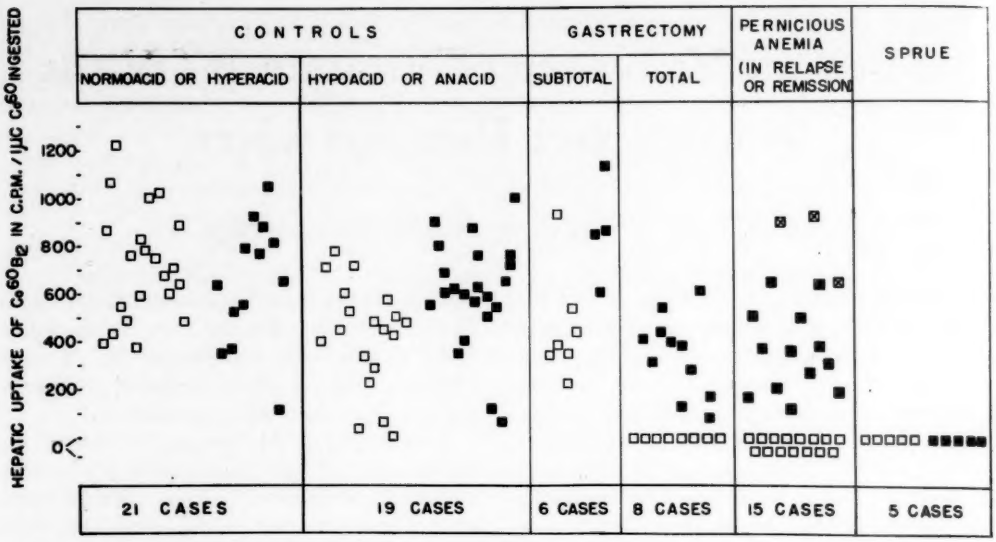

FIG. 1. Uptake of radioactive vitamin B_{12} by the liver in seventy-four persons following oral administration of Co^{60}-vitamin B_{12} alone (□) or together with potent intrinsic factor concentrate from the hog's stomach (■). (From: GLASS, G. B. J., PACK, G. T. and MERSHEIMER, W. L. *Gastroenterology*, 29: 666, 1955.[7])

defect in the absorptive mechanism of the small intestine.

DEPOSITION OF VITAMIN B_{12} IN THE LIVER IN HEPATIC DISEASE

Defective hepatic deposition of radioactive vitamin B_{12} may be due to hepatic disease as well. In about one-third of thirty-six patients with hepatic disease studied, the hepatic uptake of Co^{60}-vitamin B_{12} was below the lower limit of normal,[11] as shown in Figure 2. In three of them, i.e., in one patient with severe hepatitis, one with terminal liver cirrhosis, and one with advanced malignancy of the liver, the hepatic uptake was entirely abolished. No correlation could be observed, however, between the extent of damage to the liver, as shown by liver function tests, and the defective hepatic deposition of vitamin B_{12}.

The addition of intrinsic factor does not improve the hepatic uptake of radioactive vitamin B_{12} in hepatic disease. It seems reasonable to conclude, therefore, that the impaired hepatic uptake is due to damage to the liver cells, by which the ability of the liver cells to accept and deposit vitamin B_{12} has become impaired. This is supported by our other findings which indicate that the deposition of radioactive vitamin B_{12}, following parenteral administration, may be impaired in some of these cases as well.[11]

Vitamin B_{12} blood levels in advanced hepatic disease are, as a rule, high.[12–15] This has been correctly related to the liberation of hepatic vitamin B_{12} into the blood following destruction of liver tissue. Our findings suggest that high blood levels in some of these patients may also be due, in part, to the inability of the damaged liver cells to remove the vitamin from the circulation.

TURNOVER OF VITAMIN B_{12} IN DOGS

We next investigated the hepatic turnover rate of vitamin B_{12} in normal dogs.[16] These animals received a single injection of radioactive vitamin B_{12}, following which, for an observation period of as long as ten months, we determined the radioactivity of their livers by external recording. The tracings in the semi-logarithmic plot of Figure 3 represent the discharge of Co^{60} from dogs' livers, as determined by hepatic surface radioactivity. The arrows indicate the biologic half-life of radioactive vitamin B_{12} in the liver. This varied

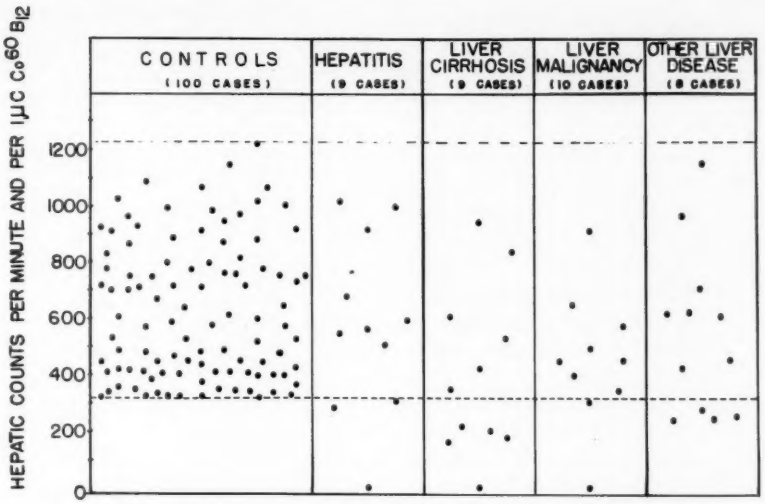

FIG. 2. Hepatic uptake of radioactive Co^{60}-vitamin B_{12} in 100 control subjects and thirty-six patients with hepatic disease. (From: GLASS, G. B. J., BOYD, L. J. and EBIN, L. *J. Lab. & Clin. Med.*, 52: 849, 1958.[11])

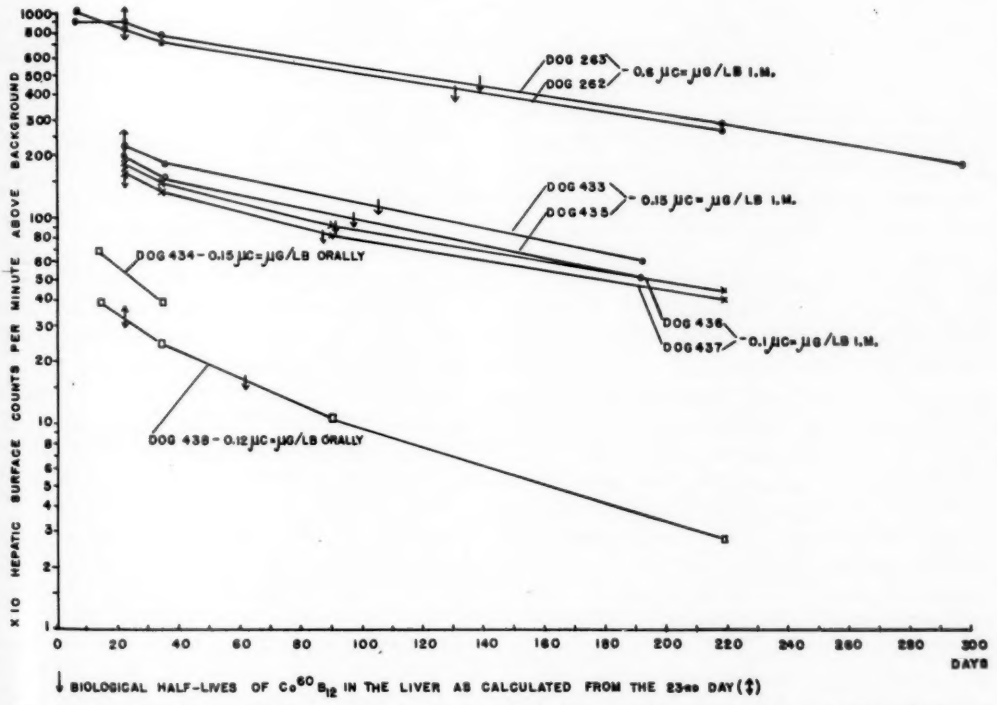
X 10 HEPATIC SURFACE COUNTS PER MINUTE ABOVE BACKGROUND
DOG 263
DOG 262
- 0.6 μC = μG/LB I.M.
DOG 433
DOG 435
- 0.15 μC = μG/LB I.M.
DOG 434 - 0.15 μC = μG/LB ORALLY
DOG 436
DOG 437
- 0.1 μC = μG/LB I.M.
DOG 438 - 0.12 μC = μG/LB ORALLY
DAYS
BIOLOGICAL HALF-LIVES OF Co^{60} B_{12} IN THE LIVER AS CALCULATED FROM THE 23rd DAY (‡)

FIG. 3. Hepatic surface counts in eight dogs followed up to ten months after parenteral or oral administration of radioactive Co^{60}-vitamin B_{12}. (From: GLASS, G. B. J. and MERSHEIMER, W. L. *J. Lab. & Clin. Med.*, 52: 860, 1958.[16])

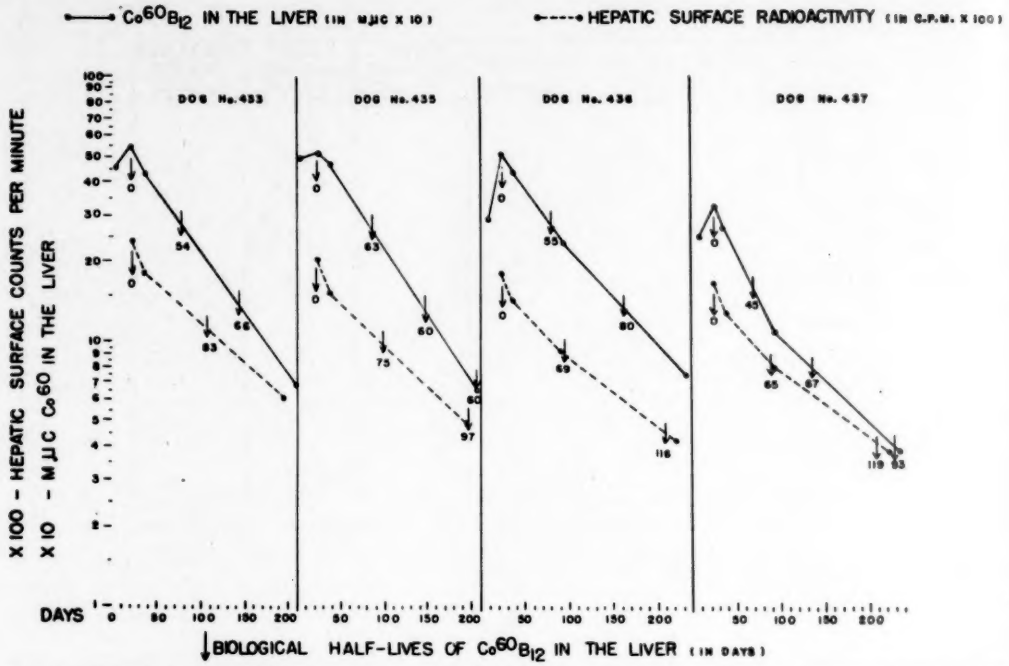
Co60B12 IN THE LIVER (IN MμC X 10)
HEPATIC SURFACE RADIOACTIVITY (IN C.P.M. X 100)
X 100 - HEPATIC SURFACE COUNTS PER MINUTE
X 10 - MμC Co60 IN THE LIVER
DOG No. 433
DOG No. 435
DOG No. 436
DOG No. 437
DAYS
BIOLOGICAL HALF-LIVES OF Co60B12 IN THE LIVER (IN DAYS)

FIG. 4. Hepatic storage of Co^{60}-vitamin B_{12} and its discharge from the liver, as determined by surgical biopsies and hepatic surface scanning. (From: GLASS, G. B. J. and MERSHEIMER, W. L. *J. Lab. & Clin. Med.*, 52: 860, 1958.[16])

from six to twelve weeks in our dogs, depending on the dose injected. The discharge of vitamin B_{12} from the dog's liver proceeds much faster than it does in human beings, probably because of a more rapid general metabolic turnover.

At the bottom of Figure 3 is the tracing of the hepatic radioactivity following oral administration of Co^{60}-vitamin B_{12}. This is much lower than that obtained after a similar dose of radioactive vitamin B_{12} given intramuscularly, shown in the middle of this figure. This indicates the limited range of intestinal absorption of vitamin B_{12} in dogs, a limitation similar to that found in human beings.[17,18]

In four dogs, in addition to the measurement of surface radioactivity, we determined the radioactivity in the liver directly, by serial surgical biopsies of the liver (Fig. 4). These were repeated at intervals of several weeks for the duration of the observation period. The measurements of vitamin B_{12} in specimens obtained by surgical biopsies correlated well with the concentration of radioactive vitamin B_{12} in the entire homogenized organ.

The upper tracings of Figure 4, shown by continuous lines, represent the radioactivity of the liver as determined by serial surgical biopsies. These tracings are compared with those of hepatic surface radioactivity, shown below by intermittent lines. The consecutive biologic half-lives of radioactive vitamin B_{12} in the liver are again shown by arrows. The correlation obtained by both technics was close, and the tracings obtained were grossly parallel. The determination of the biologic half-lives of vitamin B_{12} in the liver by both methods gave similar values, in the range of from six to twelve weeks.

The distribution of Co^{60}-vitamin B_{12} in the viscera of two dogs was studied postmortem, several months after a single injection of Co^{60}-vitamin B_{12}. The concentration of radioactivity in the organs was highest in the kidneys, next highest in the liver, and followed in descending order, by the pancreas, spleen, small intestine and lungs. This is in line with the observations of Willigan et al.[19] who showed that the kidney of the dog contains a

TABLE I

Content of Radioactivity in the Liver and Kidneys of Five Dogs Sacrificed 205 to 232 Days After Intramuscular Administration of Co^{60}-Vitamin B_{12}

Dog	Dose of Co^{60}-Vitamin B_{12} Injected in μc./lb. Body Weight	Day after Administration	Radioactivity in mμc./gm. Tissue and per μc./lb. Body Weight Injected	
			Liver	Kidneys
1	0.10	232	0.30	0.37
2	0.10	232	0.37	0.35
3	0.15	205	0.20	0.35
4	0.15	205	0.27	0.25
5	0.60	218	0.14	0.20

higher concentration of vitamin B_{12} than does the liver.

The total vitamin B_{12} content in the viscera is highest in the liver, which contains more of the vitamin than all other abdominal organs combined. The kidneys are the second main storage depot, followed by the pancreas, small intestine, spleen and lungs.

The data in Table I show that larger parenteral doses of vitamin B_{12} result in much higher losses and less efficient retention in the organs. We calculated the radioactivity in the liver and kidneys per gram of tissue and per 1 μc. of Co^{60}-vitamin B_{12} injected per pound of the animal's weight. The larger the injected dose, the less, on a percentage basis, of the material injected will be stored in the liver and kidneys. In other words, one can "load" the liver with vitamin B_{12} much more effectively if one gives the same dose in small fractional portions at repeated intervals rather than as a single, massive dose. This has been thought to be so for a long time, but our data now give it experimental support.

RADIOACTIVE VITAMIN B_{12} IN THE LIVER

The data contained in textbooks on isotopes and in the publications of the Atomic Energy Commission mention a figure of 68 per cent as the total radioactivity of the body stored in the liver as the target organ, after administration of Co^{60}.[20] We believe that this figure is incorrect, not only for dogs, but for human beings as well. At the peak of hepatic radioactivity, as determined by biopsy of the liver, the amount of radioactivity found in the liver of dogs (after intramuscular administration of 0.1 to 0.15 μg. of radioactive vitamin B_{12}, which corresponds to about 15 μg. in man) is from 18 to 27 per cent of the administered dose. This figure is much less than the 68 per cent just mentioned. Our belief is that the same is true as well for human beings.

We attempted to determine whether the prolonged hepatic radioactivity following administration of Co^{60}-vitamin B_{12} is, in fact, due to hepatic storage of radioactive vitamin B_{12}, or whether it is due, rather, to the deposition of the radioactive cobalt atom in the liver.*

A slurry of the liver of a dog sacrificed seven months after injection of radioactive vitamin B_{12} was processed into a cyanocobalamin concentrate by the standard cyanide-nitrite treatment, followed by extraction by benzyl alcohol-ethyl ether and methanol. The usual recovery of cyanocobalamin by this procedure is in the range of 60 to 70 per cent of the total content of vitamin B_{12} in the liver. The recovery of radioactivity was in a similar range, 60 to 62 per cent. This shows that most of the radioactivity which accumulates in the liver after administration of radioactive vitamin B_{12} passes into the cyanocobalamin extract, and also indicates that the radioactivity was in association with the vitamin.

The cyanocobalamin extract was then submitted to descending chromatography in secondary butanol on large channelled sheets of filter paper for thirty hours. The papers were then cut into horizontal strips, one inch wide. Some strips were eluted, and the vitamin B_{12} content in the elutes was determined by Lactobacillus leichmannii and Euglena gracilis assay. In the other cut strips, the radioactivity was measured in a well counter.

Only small amounts of microbiologic activity and radioactivity accumulated in the zone of origin, the factor-B zone, and in the intermediate area. In contrast, about 85 per cent

* This work was done in association with Drs. F. H. Wolf and R. H. Weston of Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania.

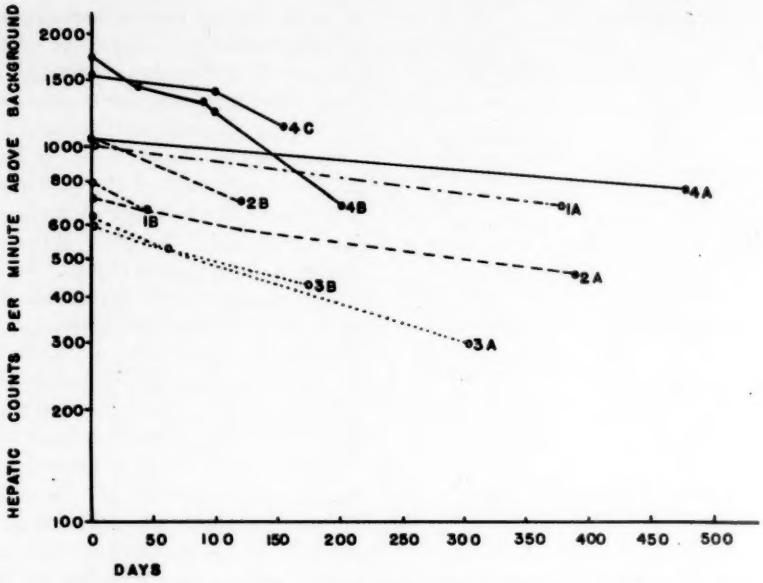

FIG. 5. Biologic decay of radioactivity over the liver in one patient with nutritional anemia (No. 1) and in three patients with pernicious anemia (Nos. 2 through 4), as determined by surface scintillation counting. (From: GLASS, G. B. J. *J. Lab. & Clin. Med.*, 52: 875, 1958.[27])

of the microbiologically active vitamin B_{12}, and up to 63 per cent of the total radioactivity, were located in a single zone, that corresponding to the usual area of cyanocobalamin.

This clearly shows that, seven months after administration of a single dose of radioactive vitamin B_{12}, at least 60 per cent of the hepatic radioactivity emanates from the microbiologically active, labeled vitamin B_{12} stored in the liver.

DISCHARGE OF RADIOACTIVE VITAMIN B_{12} FROM THE LIVER IN MAN

When a single dose of labeled vitamin B_{12} is injected intramuscularly into a human subject,[21] the radioactivity over the site of the injection decreases rapidly with the result that, four hours later, not more than about 1 per cent of the initial activity remains. A few minutes after injection some radioactivity appears over the cutaneous projections of the liver, spleen and kidneys and this gradually increases during the next few hours.

Subsequently, the radioactivity over the liver continues to increase over the next five to seven days, while that over the kidneys and spleen levels off or diminishes.[21] This is probably due to a relocation of vitamin B_{12} at this time from other tissues to the liver. After seven to ten days, however, the hepatic radioactivity levels off, and then for a period of months decreases only slightly.[21] The human liver is thus shown to retain vitamin B_{12} most tenaciously, like the animal's liver,[1-4] a finding in man first reported by Smith[22] and since confirmed by many other investigators.[21,23-26]

Table II summarizes our results in eighteen subjects studied for a period of forty-five to 878 days.[27] The data show that only about 5 per cent, on the average, of the hepatic stores of vitamin B_{12} are discharged each month from the liver of both normal subjects and patients with pernicious anemia in remission. Large individual variations are observed in this figure however; the range in various subjects extending from 1.5 per cent to about 10.5 per cent per month.[27] The slopes of the tracings

TABLE II

Rate of Discharge of Co^{60}-Vitamin B_{12} from the Liver in Ten Control Subjects and Eight Patients with Pernicious Anemia in Remission

Group	No. of Cases	No. of Observations	Duration of Observation Period (days)			Decrease in Hepatic Surface Radioactivity per Month (%)			Presumptive Biologic Half-Life Time of Co^{60}-Vitamin B_{12} in the Liver (mo.)		
			Range	Mean	Median	Range	Mean	Median	Range	Mean	Median
Control subjects	10	11	45–377	139	101	2.4–10.5	5.5	4.1	4.8–20.8	11.1	12.1
Patients with pernicious anemia in remission	8	12	64–878	297	214	1.5–10.3	4.8	4.9	4.8–33.3	14.5	11.9
Totals	18	23	45–878	222	169	1.5–10.5	5.1	4.2	4.8–33.3	12.8	12.0

are not parallel, however, which suggests individual differences among various subjects in the rate of hepatic discharge (Fig. 5). It is to be noted, moreover, that the rate of discharge from the liver may change at various times in the same person.

CONCLUSIONS

Hepatic surface radioactivity is a true measure of the content of radioactive vitamin B_{12} in the liver. This assay technic can also be applied successfully to the measurement of the intestinal absorption of vitamin B_{12} and to the diagnosis of pernicious anemia and sprue in relapse and remission.

In one-third of patients with hepatic disease, the ability of the liver cells to take up vitamin B_{12} is impaired. This may be a contributory factor in causing the high vitamin B_{12} blood levels in some cases of hepatitis, liver cirrhosis and malignant infiltration of the liver.

The liver is the main storage organ for vitamin B_{12} in man and the dog. At least 60 per cent of the radioactivity found in the liver long after the administration of a single dose of radioactive vitamin B_{12} is due to the microbiologically active labeled vitamin B_{12} which has been stored in the liver.

The rate of discharge of the vitamin from the liver of the dog is about five times faster than it is in human beings. The rate of discharge from the liver in man is from 1.5 to 10 per cent per month, which assumes that the biologic half-life of vitamin B_{12} in the human liver is, on the average, one year, with individual variations of from five to thirty-three months.

This remarkably prolonged period of hepatic storage of vitamin B_{12} in man explains the long latent periods which precede the development of clinical vitamin B_{12} deficiencies.

REFERENCES

1. Barbee, K. W. and Johnson, B. C. Metabolism of radioactive vitamin B_{12} by the rat. *Proc. Soc. Exper. Biol. & Med.*, 76: 720, 1951.
2. Rosenblum, C., Chow, B. F., Condon, G. P. and Yamamoto, R. S. Oral versus parenteral administration of Co^{60}-labeled vitamin B_{12} to rats. *J. Biol. Chem.*, 198: 915, 1952.
3. Miller, A., Gaull, G., Ross, J. F. and Lemon, H. M. Tissue distribution of parenteral Co^{60} vitamin B_{12} in mouse, hamster, rat and guinea pig. *Proc. Soc. Exper. Biol. & Med.*, 93: 33, 1956.
4. Wolff, R. La position du foie dans le métabolisme normal et pathologique de la vitamin B_{12}. In: Heinrich. H. C. Vitamin B_{12} and Intrinsic Factor. p. 519. Stuttgart, 1957. Ferdinand Enke.
5. Glass, G. B. J., Boyd, L. J., Gellin, G. A. and Stephanson, L. Uptake of radioactive vitamin B_{12} by the liver in humans: test for measurement of intestinal absorption of vitamin B_{12} and intrinsic factor activity. *Arch. Biochem.*, 51: 251, 1954.
6. Glass, G. B. J. and Boyd, L. J. Differentiation of macrocytic anemias and diagnosis of pernicious anemia and sprue in remission by accelerated

measurement of hepatic uptake of radioactive Co^{60} B_{12}. *Ann. Int. Med.*, 47: 274, 1957.

7. Glass, G. B. J., Pack, G. T. and Mersheimer, W. L. Uptake of radioactive vitamin B_{12} by the liver in patients with subtotal and total gastrectomy. *Gastroenterology*, 29: 666, 1955.
8. Glass, G. B. J. The role of the gastro-intestinal tract in the pathogenesis and management of pernicious anemia. *Bull. New York M. Coll.*, 16: 1, 1953.
9. Glass, G. B. J. and Boyd, L. J. Diagnosis of oligosymptomatic forms of sprue by measurement of the hepatic uptake of radioactive vitamin B_{12}. In: Proceedings of the Fifth Pan-American Congress of Gastroenterology, vol. 2, p. 967. Havana, 1956. Fernandez y Cia.
10. Glass, G. B. J. Intestinal absorption and hepatic uptake of vitamin B_{12} in diseases of the gastrointestinal tract. *Gastroenterology*, 30: 37, 1956.
11. Glass, G. B. J., Boyd, L. J. and Ebin, L. Radioactive vitamin B_{12} in the liver. I. Hepatic uptake of Co^{60} B_{12} in liver disease. *J. Lab. & Clin. Med.*, 52: 849, 1958.
12. Wolff, R., Royer, P. and Karlin, R. Serum vitamin B_{12} in experimental liver injury. *Bull. Soc. chim. biol.*, 34: 538, 1952.
13. Rachmilewitz, M., Aronovitch, J. and Grossovicz, N. Serum concentrations of vitamin B_{12} in acute and chronic liver disease. *J. Lab. & Clin. Med.*, 48: 339, 1956.
14. Kristensen, H. P. O. The vitamin B_{12} concentration in plasma from patients with acute hepatitis and obstructive jaundice. *Nord. med.*, 55: 85, 1956.
15. Jones, P. N. and Mills, E. H. The effect of liver disease on serum vitamin B_{12} concentrations. *J. Clin. Invest.*, 35: 717, 1956.
16. Glass, G. B. J. and Mersheimer, W. L. Radioactive vitamin B_{12} in the liver. II. Hepatic deposition, storage and discharge of Co^{60} B_{12} in dogs. *J. Lab. & Clin. Med.*, 52: 860, 1958.
17. Glass, G. B. J., Boyd, L. J. and Stephanson, L. Intestinal absorption of vitamin B_{12} in humans as studied by isotope technic. *Proc. Soc. Exper. Biol. & Med.*, 86: 522, 1954.
18. Swendseid, M. E., Gasster, M. and Halsted, J. A. Limits of absorption of orally administered vitamin B_{12}: effect of intrinsic factor sources. *Proc. Soc. Exper. Biol. & Med.*, 86: 834, 1954.
19. Willigan, D. A., Meyer, L. M. and Cronkite, E. P. The distribution and turnover of parenterally injected Co^{60}-labeled vitamin B_{12}. *Clin. Res. Proc.*, 5: 144, 1957.
20. Rosenblum, C. Radiation comparison of cobalt isotopes. *Am. J. Clin. Nutrition*, 8: 276, 1960.
21. Glass, G. B. J., Boyd, L. J. and Gellin, G. A. Surface scintillation measurements in humans of the uptake of parenterally administered radioactive vitamin B_{12}. *Blood*, 10: 95, 1955.
22. Smith, E. L., Gurney, D. M. and Howat, A. G. In: Second International Congress of Biochemistry, p. 19. Paris, 1952.
23. Meyer, L. M., Arkun, S. N. and Jiminez-Casado, M. Further observations on distribution of radioactivity, following parenteral administration of Co^{60} vitamin B_{12}. *Proc. Soc. Exper. Biol. & Med.*, 92: 515, 1956.
24. Meyer, L. M., Berlin, N. I., Jiminez-Casado, M, and Arkun, S. N. Vitamin B_{12} distribution determined by surface body counting following parenteral administration of Co^{60} vitamin B_{12}. *Proc. Soc. Exper. Biol. & Med.*, 91: 129, 1956.
25. Schloesser, L. L. and Schilling, R. F. Biologic half-life of hepatic radioactivity from vitamin B_{12} Co^{60}. Presented at the International Society of Hematology, Sixth Congress, p. 245. Boston. August, 1956.
26. Doscherholmen, A. and Hagen, P. S. Alteration of hepatic storage of radiolabeled vitamin B_{12}. *Proc. Soc. Exper. Biol. & Med.*, 95: 667, 1957.
27. Glass, G. B. J. Radioactive vitamin B_{12} in the liver. III. Hepatic storage and discharge of Co^{60} B_{12} in pernicious anemia. *J. Lab. & Clin. Med.*, 52: 875, 1958.

The Mode of Action of Intrinsic Factor in Vitamin B_{12} Absorption by Rat Intestine

RENÉ L. WOLFF, M.D. AND JANINE VUILLEMIN-WEIS, B.S.

LITTLE is known about the mode of action of intrinsic factor in vitamin B_{12} absorption by rat intestine. In order to obtain some information about this problem, we have studied the intestinal and hepatic uptake of this vitamin at different intervals of time (from thirty minutes up to seventy-two hours after intubation) in adult white rats receiving 100 mμg. of Co^{60}-vitamin B_{12} by gastric intubation. Identical studies were made in totally gastrectomized rats, and the results of this comparative study are given here.

MATERIAL AND METHODS

The experiments were made with a rat gastric-mucosal extract, which was prepared by homogenizing the gastric mucosa in a Potter Elvejhem homogenizer in twelve parts of saline. The homogenate was centrifuged to give a clear solution. After repeated freezing and thawing of this solution, small amounts of insoluble matter could be separated. The final solution no longer contained insoluble material.

After severing the ligated part of the intestine and after thorough washing with saline, the whole intestine was dissolved in NaOH solution by heating in a water bath. The tube containing the intestine material was inserted into a well scintillation counter.

Total gastrectomy was performed according to the procedure of Machella and Griffith[1] and of Nieweg et al.,[2] as modified by Nabet et al.[3] The small intestine, not including the duodenum, was excised from the rat and submitted to three washings of thirty minutes each, with 50 ml. of Krebs glucose-phosphate medium at 37°C., in the Warburg apparatus. The purpose of the washings was to eliminate traces of vitamin B_{12} solution retained by capillarity between the intestinal villi. A preliminary control study afforded evidence that oxygen uptake was not altered by this procedure and that during these washings unbound vitamin B_{12} gradually disappeared.

Radioactivity measurements were performed on the Co^{60} salt, obtained by wet washing of the intestine, corrections being allowed for self-absorption and background countings. The use of a windowless flow counter ensured satisfactory recoveries with low counting rates.

RESULTS

The results obtained on normal and gastrectomized rats are given in Table I.

Two stages in the uptake of the vitamin B_{12} by rat intestine at various time intervals may be differentiated. In the first stage, there is a rapid increase of vitamin B_{12} uptake followed by a rapid* but incomplete decrease which slackens from the third to the seventy-second hour after oral administration.

The first stage (rapid increase) bears no relationship to the evolution of the hepatic Co^{60}-vitamin B_{12}. In the second stage, the slow decrease of intestinal Co^{60}-vitamin B_{12} is accompanied by an hepatic uptake of this vita-

From the Department of Biochemistry, Faculty of Medicine, Nancy, France.

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* In normal rats, Booth et al.[4] observed a similar maximal uptake of vitamin B_{12} in the small intestine (between fifteen minutes and two hours after the oral dose).

TABLE I

Intestinal and Hepatic Uptake of Co^{60}-vitamin B_{12} After Oral Intubation in Normal and Gastrectomized Rats

Time Elapsed (hr.)	Normal Rats	Gastrectomized Rats	Gastrectomized Rats Plus Intrinsic Factor
	Intestinal Uptake of Vitamin B_{12} ($m\mu g.$)		
1/2	2.90 (1)	...	...
1	12.06 ± 3 (7)	5.66 (1)	...
3	1.46 ± 0.28 (5)	0.10 (1)	...
24	1.36 ± 0.43 (6)	0.105 ± 0.035 (2)	0.38 (1) 1.00 (1)
48	1.20 ± 0.25 (7)	...	...
72	0.80 ± 0.34 (5)	...	...
	Hepatic Uptake of Vitamin B_{12} ($m\mu g.$)		
1/2	0.08 (1)	...	...
1	0.08 ± 0.004 (7)	0.12 (1)	...
3	0.77 ± 0.13 (5)	0.35 (1)	...
24	1.97 ± 0.47 (6)	0.13 ± 0.03 (2)	0.35 (1) 1.75 (1)
48	2.40 ± 0.25 (7)	...	...
72	2.72 ± 0.24 (5)	...	...

* Numbers between parentheses indicate the number of animals. The results are given with the standard error of the mean.

min in amounts of the same magnitude as those which disappear from the gut. These results visualize the moving of the vitamin B_{12} from the intestinal mucosa to the liver.

In gastrectomized rats, the first stage is also observed although to a lesser degree. In the second stage, extending from the third to the seventy-second hour, the previously absorbed Co^{60}-vitamin B_{12} disappears almost completely from the intestine.

From these results, it may be inferred that intrinsic factor does not interfere with the intestinal uptake of vitamin B_{12} but that it is essential for the retention of the absorbed vitamin in the gut.

In normal animals, a part of the absorbed vitamin B_{12} is retained in the intestine; from there it is removed to the liver. In gastrectomized rats, the intestine is no longer able to retain the vitamin and it flows back to the intestinal lumen from where it is excreted by the feces.

In the liver of gastrectomized rats, in contrast to what occurs in normal animals, no increase of Co^{60}-vitamin B_{12} may be noted. The amount of vitamin B_{12} in the liver remains low.

In gastrectomized animals, receiving intrinsic factor by oral administration, the intestinal as well as the hepatic uptake of vitamin B_{12} after twenty-four hours tends to become normal.

CONCLUSION

These results are in accord with the fact that the intestinal uptake of orally administered vitamin B_{12} is not dependent on the presence of intrinsic factor, but that its role is essentially that of retaining the already absorbed vitamin B_{12} in the intestinal mucosa and preventing it from overflowing into the intestinal lumen and being excreted by the feces.

REFERENCES

1. Machella, T. H. and Griffith, J. Q., Jr. The Rat in Laboratory Investigation, p. 168. Philadelphia and London, 1949. J. B. Lippincott Co.
2. Nieweg, H. O., Arends, A., Mandema, E. and Castle, W. B. Enhanced absorption of vitamin B_{12} in gastrectomized rat by rat intrinsic factor. *Proc. Soc. Exper. Biol. & Med.*, 91: 328, 1956.
3. Nabet, P., Arnould, P. and Wolff, R. La gastrectomie totale chez le rat. *J. Physiol.*, 50: 91, 1958.
4. Booth, C. C., Chanarin, I., Anderson, B. B. and Mollin, D. L. The site of absorption and tissue distribution of orally administered 56Co labelled vitamin B_{12} in the rat. *Brit. J. Haemat.*, 3: 253, 1957.

The Uptake of Radioactive Vitamin B_{12} by Perfused Rat Intestine *In Situ*

RENÉ L. WOLFF, M.D.

PREVIOUS work on gastrectomized rats has demonstrated that intestinal uptake of vitamin B_{12} takes place only when the intrinsic factor preparation of the rat is administered by intubation at the same time as vitamin B_{12}.[1] Under these circumstances, it is possible to observe vitamin B_{12} absorption by the intestinal mucosa in as little as twelve to fifteen minutes.

In a study of *in situ* absorption of vitamin B_{12} by the small intestine of normal rats (severed at the stomach and cecum by ligature), Holdsworth and Coates[2] in an eighteen to twenty-four–hour study observed the intrinsic factor-controlled absorption of this vitamin. This was demonstrated by the amount of vitamin B_{12} in the cecum and the liver and, more recently, in an intestinal loop.[3]

Upon examination of the results of these two experimental procedures, it appeared that, in the latter, a rapid intrinsic factor-dependent uptake of vitamin B_{12} might possibly occur. A short time experimental procedure on normal rats, analogous to that outlined by Holdsworth and Coates, but extending over no more than a two-hour period (the duration of a Nembutal® anesthesia), was likely to ensure satisfactory results.

This proved to be true. In the absence of intrinsic factor, the intestinal uptake amounts to an average of 1.7 per cent of the administered dose of vitamin B_{12}, and in the presence of intrinsic factor, the uptake increases to an average of 17 per cent.

From the Department of Biochemistry, Faculty of Medicine, Nancy, France.

Presented at the Vitamin B_{12} Symposium, sponsored by the Society for the Study of Blood, April 11, 1958, at the New York Medical College, New York, New York.

EXPERIMENTAL PROCEDURE

Perfusion

An intraperitoneal injection of Nembutal was given to rats weighing 200 to 300 gm. that had fasted twenty-four hours. Anesthesia occurred rapidly and lasted from one and a half to two hours. When necessary, a second injection with half the amount of Nembutal was given after one and a half hours to ensure complete anesthesia for at least two hours.

The lower portion of the abdomen was opened by a midline incision and the small intestine was ligated 2 to 3 cm. from the pylorus in the vicinity of the cecum. At the two ends, the intestinal wall was opened and a glass canula was inserted into each end. The abdomen was then closed with clips and the intestine was perfused with 100 ml. of 37°C. saline. The perfusion was controlled by a drip device and the saline preheated in a thermostat. A washing of about ten minutes was ample to deplete the intestine of traces of intrinsic factor.

After closing the rubber tubing with a clamp at each end, a mixture of 1 ml. of a known dilution of radioactive vitamin B_{12} and 1 ml. of rat gastric mucosa preparation was injected through the proximal rubber tubing into the intestinal lumen. This was followed by 1 ml. of saline. The solution remained in the intestinal lumen for forty-five minutes.

Following that time, a slow perfusion of 50 ml. of saline was started and continued over a period of thirty minutes to distribute the vitamin B_{12} intrinsic factor solution along the intestinal wall.

This procedure was followed by the removal of all traces of vitamin B_{12} remaining in the intestinal lumen or by capillarity in the interstices of the villi by three rapid washings per-

TABLE I
Intestinal Uptake of Co^{60}-Vitamin B_{12} Alone and in the Presence of Rat Stomach Mucosa

Per cent Vitamin B_{12} Alone (8 rats)	Per cent Vitamin B_{12} Intrinsic Factor (17 rats)	
2.48	28.58	7.42
3.44	5.83	18.80
2.14	13.10	34.70
0.88	4.86	12.50
0.63	31.70	21.30
1.23	19.10	22.20
1.13	26.30	22.36
1.93	14.45	26.90
		22.90
1.73 ± 0.293*	20.76 ± 8.94*	

* Average.

formed with 50 ml. of saline, each for a duration of five to seven minutes. All perfusion liquids were collected separately for control.

The rat was then sacrificed and the intestine was severed distal to the pylorus and removed from the animal. All superfluous mesentery and fat were removed. The intestine was opened along the line of mesenteric attachment, washed three times in 0.9 per cent sodium chloride and divided into small sections. The whole intestine was put into a glass tube, dissolved by heating the tube in a water bath in the presence of 1 ml. of concentrated sodium hydroxide, and then inserted into a scintillation well counter.

Correction was provided for background and the height of the solution in the tube. The results were calculated in per cent of the amount of administered vitamin B_{12} taken up by the intestine.

Solution of Co^{60} Labeled Vitamin B_{12}

Radioactive vitamin B_{12} was obtained by a fiftyfold dilution from a standard solution of Merck & Co., the concentration of which was 1 ml. = 21 mγ and the specific activity 1 μc. per ml. Each milliliter of this diluted solution had 13,000 counts per minute.

Intrinsic Factor

The supernatant of a centrifuged homogenate in saline of the mucosal part of rat intestine was used. The suspension was obtained by homogenizing twelve glandular parts of rat stomach in 60 ml. of saline, in a Potter-Elvejhem homogenizer. One milliliter of the supernatant corresponded to 0.2 rat stomach. Table I shows the results obtained.

COMMENTS

There is a considerable difference between the amount of vitamin B_{12} taken up by the rat intestine in the absence of intrinsic factor and in the presence of intrinsic factor. Whereas the scattering is small in the intrinsic factor-depleted intestine, it becomes large in the presence of intrinsic factor. The irregular gradient concentration of vitamin B_{12} and intrinsic factor along the intestinal wall, and the difficulty to warrant reproducibility, may, at least in part, be responsible for this irregularity. Even with such a wide scattering there is no example of overlapping, and it seems likely that with a statistical mean obtained on four animals, a clear-cut effect of intrinsic factor may appear.

We hope to quantitate the action of intrinsic factor in a more reproducible procedure.

CONCLUSION

The small intestine of normal rats, isolated by ligature and maintained *in situ* with all its connections, and depleted from intrinsic factor by perfusion, takes up added vitamin B_{12} in an amount which depends greatly on the added intrinsic factor.

A method is described whereby rat intrinsic factor activity of gastric mucosal extract may be demonstrated in a two-hour test on normal anesthetized rats.

REFERENCES

1. Nieweg, H. O., Shen, S. C. and Castle, W. B. Mechanism of intrinsic factor action in the gastrectomized rat. *Proc. Soc. Exper. Biol. & Med.*, 94: 223, 1957.
2. Holdsworth, E. S. and Coates, M. E. Binding factors in the absorption of vitamin B_{12} by the rat. First European Symposium on Vitamin B_{12} and Intrinsic Factor, pp. 241–243. Hamburg, 1957.
3. Holdsworth, E. S. and Coates, M. E. The absorption of vitamin B_{12} in the rat. *Biochem. J.*, 69: 20P, 1958.

The Relation of Binding Power to Intrinsic Factor Activity

Effect of Pseudovitamin B_{12} on Absorption of Vitamin B_{12}

MILTON TOPOREK, PH.D.*

THE QUESTION of whether or not "binding" was related to intrinsic factor activity has been under investigation for many years. In 1950, Prusoff and co-workers[1] concluded that binding had no relationship to intrinsic factor activity. Their statement was based on results that showed that intrinsic factor concentrates with the lowest "binding power" gave them the best vitamin B_{12} absorption under the conditions of their experiments. In 1954, Williams, Ellenbogen and Esposito[2] also stated that vitamin B_{12} binding was probably not a property of intrinsic factor. The validity of these statements and the experiments upon which they were based could appropriately be questioned on the basis of: (1) non-uniformity of the methods used to prepare the experimental intrinsic factor concentrates; (2) non-uniformity in activity of concentrates made exactly the same way; (3) lack of definitive knowledge concerning the composition and effects of the large fraction of impurities contaminating the intrinsic factor concentrates; (4) possible changes from the natural state of intrinsic factor as it finally appears in the concentrates; and (5) a lack of an adequate or uniform definition of binding power.

From the Thomas Henry Simpson Memorial Institute for Medical Research, The University of Michigan, Ann Arbor, Michigan.

* Present address: Assistant Professor of Biochemistry, Department of Biochemistry, Jefferson Medical College, Philadelphia, Pennsylvania.

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In 1955 a number of publications appeared[3–5] from which it could be inferred that the pendulum was swinging in the direction of the possibility that binding was a necessary property of intrinsic factor activity. This report is not intended as a complete review of the literature but should serve to indicate some of the pertinent highlights. At approximately this time, it appeared from observations in this laboratory resulting from an extensive program of testing intrinsic factor preparations that binding was definitely essential for intrinsic factor activity. This paper presents the experimental procedure and results supporting the previously published preliminary reports[6,7] of the work carried out to prove this point. The urinary excretion test essentially as described by Schilling[8] was used as a measure of vitamin B_{12} absorption.

EXPERIMENTAL METHODS

Source of Intrinsic Factor: To circumvent most of the criticisms inherent to previous approaches to this problem of determining the relation of binding to intrinsic factor activity, pooled normal human gastric juice (NHGJ) was used as the source of intrinsic factor in the experiments to be described. After collection, the NHGJ was filtered through several layers of cheese cloth, adjusted to pH 7, and then frozen until used, thus giving a product only slightly removed from its native physiologic state.

Binding: When referred to with respect to the experiments described in this paper, binding means that property of the intrinsic

TABLE I

Net Percentage of Oral Dose of Co^{60}-Vitamin B_{12} Excreted by Totally Gastrectomized Patients in Twenty-Four–Hour Urine Collection

Patient	Mixture		
	Control	Cold	Hot
Daw	0.0	1.2	6.0
Daw	0.5	2.7	6.5
Bla	1.3	2.3	9.5
Noo	0.9	4.0	5.3
And	0.6	1.0	4.8
Mac	0.5	3.0	8.0
Kra	0.3	1.9	5.5
Gra*	1.1	1.0	4.8
Pol*	0.7	0.5	7.2

NOTE: See section on experimental methods for details regarding composition of mixtures and doses.
* Patient with pernicious anemia.

TABLE II

Net Percentage of Oral Dose of Co^{60}-Vitamin B_{12} Excreted by Patients with Pernicious Anemia in Twenty-Four–Hour Urine Collection

Patient	Mixture			
	Control Plus	(?)	Hot	Cold
Goe	B_{12} 2.7	14.9	12.4	5.7
Cla	P_s-B_{12} 2.7	13.7	17.6	0.2
Pet	B_{12} 0.6	11.6	13.1	5.4

NOTE: See section on experimental methods for details regarding composition of mixtures and doses.

factor component of NHGJ which made the added vitamin B_{12} unavailable to the test organism, L. leichmannii ATCC 7830. Each gastric juice-vitamin B_{12} preparation was assayed as is, to give a determination of "free" vitamin B_{12} in the presence of intrinsic factor, and also after autoclaving (destruction of intrinsic factor), to give "total" vitamin B_{12} in the preparation.

Preparation of Test Doses: The "cold mixture" was prepared by adding sufficient, non-radioactive, vitamin B_{12} to a sample of NHGJ to completely saturate its binding capacity and leave approximately 10 per cent free in the mixture. The mixture was allowed to stand at room temperature for thirty minutes before being used. In the first series of experiments (Table I), 2.2 μg. vitamin B_{12} (0.5 μc.) was added to 50 ml. NHGJ, approximately 2 μg. being bound by the intrinsic factor in the NHGJ and 0.2 μg. remaining free. In the second series of experiments (Table II), 0.46 μg. vitamin B_{12} (0.5 μc.) was added to 10 ml. NHGJ. The "hot mixtures" were prepared in the same way except that Co^{60}-labeled vitamin B_{12} was used instead of the non-radioactive vitamin B_{12}. The "(?) mixture" in the second series of experiments (Table II) was prepared by adding 0 46 μg. non-radioactive pseudovitamin B_{12} to 10 ml. NHGJ.

Schedule of Tests: All doses were orally administered. The NHGJ mixtures were given with any available fruit juice.

In the first series of experiments, the control mixture consisted of 2.2 μg. Co^{60}-vitamin B_{12}. The cold mixture consisted of 2.2 μg. Co^{60}-vitamin B_{12} followed immediately by a mixture of 2.2 μg. non-radioactive vitamin B_{12} in 50 ml. NHGJ. The hot mixture consisted of 2.2 μg. non-radioactive vitamin B_{12} followed immediately by a mixture of 2.2 μg. Co^{60}-vitamin B_{12} in 50 ml. NHGJ (Table I).

In the second series of experiments, the control mixture consisted of 0.46 μg. Co^{60}-vitamin B_{12} plus 0.46 μg. non-radioactive vitamin B_{12} or non-radioactive pseudovitamin B_{12}. The (?) mixture consisted of 0.46 μg. Co^{60}-vitamin B_{12} followed immediately by a mixture of 0.46 μg. non-radioactive pseudovitamin B_{12} in 10 ml. NHGJ. The hot mixture consisted of 0.46 μg. non-radioactive pseudovitamin B_{12} followed immediately by a mixture of 0.46 μg. Co^{60}-vitamin B_{12} in 10 ml. NHGJ. The cold mixture consisted of 0.46 μg. Co^{60}-vitamin B_{12} followed immediately by a mixture of 0.46 μg. non-radioactive vitamin B_{12} in 10 ml. NHGJ.

All tests were spaced one week apart to avoid any extraneous effects due to residual vitamin B_{12}.

Test Patients: The patients in series 1 had undergone total gastrectomy and were chosen to circumvent any controversy concerning binding power of their own gastric juice. Two patients with pernicious anemia were added

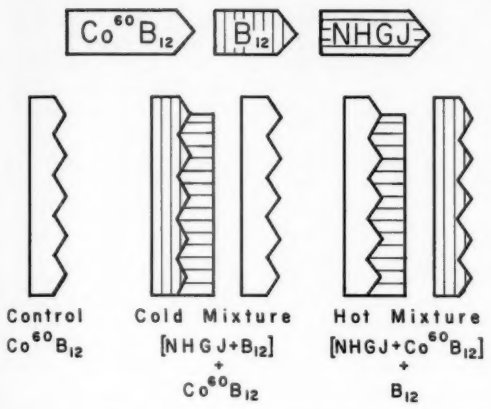

FIG. 1. Diagrammatic representation of oral test doses. *Control,* Co^{60}-labeled vitamin B_{12} only. *Cold Mixture,* normal human gastric juice with its binding capacity saturated with non-radioactive vitamin B_{12} as one component of the dose, and a second component of an equivalent amount of Co^{60}-labeled vitamin B_{12} to be taken orally, one immediately after the other. *Hot Mixture,* normal human gastric juice with its binding capacity saturated with Co^{60}-labeled vitamin B_{12} as one component of the dose, and a second component of an equivalent amount of non-radioactive vitamin B_{12} to be taken orally, one immediately after the other.

later for comparison. There was apparently no difference in response in the two types of patients. In series 2, all patients had pernicious anemia.

RESULTS

Figure 1 is a diagrammatic representation of the working hypothesis on which this work was based as well as a picture of the test doses used. The hypothesis held that if binding was a necessary property for intrinsic factor activity, that vitamin B_{12} which was bound to the intrinsic factor component of NHGJ by prior saturation of its "binding capacity," would be preferentially absorbed from the oral test mixtures presented to the patients. In other words, if the binding capacity of a sample of NHGJ was saturated by exposure to non-radioactive vitamin B_{12} and given to a patient with an equivalent amount of Co^{60}-vitamin B_{12} (cold mixture), very little Co^{60}-vitamin B_{12} would be absorbed. If, however, the binding capacity of NHGJ was saturated by exposure to Co^{60}-vitamin B_{12} and then given to a patient with an equivalent amount of non-radioactive vitamin B_{12} (hot mixture), a much greater quantity of Co^{60}-vitamin B_{12} would be absorbed. These expectations were adequately borne out (Table I). The hot mixture gave 2.5 to 14 times as much absorption as the cold mixture, with only one exception (patient Noo). These results can leave little doubt that binding, although still not adequately defined, is necessary for intrinsic factor activity. It must always be pointed out that binding by itself does not automatically indicate intrinsic factor activity, but intrinsic factor activity must carry with it the property of binding vitamin B_{12}. The possibility that the binding property may be linked with one factor and the absorption property with a second factor, intrinsic factor, does not appear to have any adequate support at the present time.

Implicit in the explanation for these results is the assumption that exchange between vitamin B_{12} bound by intrinsic factor and free vitamin B_{12} in the gastric juice mixtures would be slow or negligible. If the exchange were rapid, ratios of absorptions from hot mixture to cold mixture would be much closer to 1 rather than the values of 2.5 to 14 which were actually obtained.

Extending these experiments to the study of the effect that pseudovitamin B_{12} might have in competition for absorption with vitamin B_{12}, it was found that intrinsic factor preferentially binds vitamin B_{12} even though first exposed to pseudovitamin B_{12}. This can be seen by comparing the results obtained with the (?) mixture and the hot mixture (Table II). The absorptions measured with each mixture were almost identical. This is interpreted to mean that the binding of intrinsic factor with vitamin B_{12} is stronger than with pseudovitamin B_{12}. The ratios of absorption from hot mixture to cold mixture in this series were comparable to the ratios obtained in the first series discussed.

These results have been confirmed by the work of Bunge, Schloesser and Schilling.[9]

SUMMARY

Evidence is presented to show that binding is an essential property for intrinsic factor activity.

Exchange between bound and free vitamin B_{12} in normal human gastric juice is slow or negligible under conditions of these experiments.

Intrinsic factor preferentially binds vitamin B_{12} in the presence of pseudovitamin B_{12}.

ACKNOWLEDGMENT

The efforts of Drs. R. C. Bishop, N. A. Nelson and F. H. Bethell contributed to the success of these experiments.

REFERENCES

1. Prusoff, W. H., Meacham, G. C., Heinle, R. W. and Welch, A. D. Concentration of the intrinsic factor activity of powdered stomach. Abstracts, American Chemical Society, 118th National Meeting, p. 27A. Chicago, Illinois. September 1950.
2. Williams, W. L., Ellenbogen, L. and Esposito, R. G. Preparation of highly purified intrinsic factor. *Proc. Soc. Exper. Biol. & Med.*, 87: 400, 1954.
3. Biochemical Society Symposia, No. 13. The Biochemistry of Vitamin B_{12}, pp. 30, 49, 61, 86. London, 1955. Cambridge University Press.
4. Raine, L. The binding of vitamin B_{12} by Castle's intrinsic factor. *Nature, London*, 175: 777, 1955.
5. Baker, S. J. and Mollin, D. L. The relationship between intrinsic factor and the intestinal absorption of vitamin B_{12}. *Brit. J. Haemat.*, 1: 46, 1955.
6. Bishop, R. C., Toporek, M., Nelson, N. A. and Bethell, F. H. The relationship of binding power to intrinsic factor activity. *J. Lab. & Clin. Med.*, 46: 796, 1955.
7. Toporek, M., Bishop, R. C., Nelson, N. A. and Bethell, F. H. Comparison of the binding of pseudovitamin B_{12} and vitamin B_{12} by normal human gastric juice. Abstracts, American Chemical Society, 130th National Meeting, p. 50C. Atlantic City, New Jersey. September 1956.
8. Schilling, R. F. A new test for intrinsic factor activity. *J. Lab. & Clin. Med.*, 42: 946, 1953.
9. Bunge, M. B., Schloesser, L. L. and Schilling, R. F. Intrinsic factor studies. IV. Selective absorption and binding of cyanocobalamin by gastric juice in the presence of excess pseudovitamin B_{12} or 5,6-dimethylbenzimidazole. *J. Lab. & Clin. Med.*, 48: 735, 1956.

Symposium on Lipid Metabolism, Lipotropes and Atherosclerosis

Foreword

THIS, the first symposium of the Lipotropic Research Foundation, was held at the Sheraton Hotel, Philadelphia, Pennsylvania, on April 13, 1958. The Foundation had long considered the advisability of holding this type of meeting, but not without some degree of hesitation. Organized less than four and a half years at the time of the symposium, it was debatable as to whether or not the scope of the Foundation's activities was sufficiently broad to warrant holding such a meeting.

While the attendance was limited to invited speakers and guests, nevertheless the number in attendance and the caliber of the presentations and discussions have more than justified our decision to hold the meeting. I believe the papers presented and the discussions during the course of the meeting served the purpose intended—namely, to add to our knowledge and remove some of the confusion prevailing on the role of the lipotropes in promoting phospholipid turnover from the liver as a physiologic precursor to normal lipid transport in the blood. We hope that the publication of the proceedings of the symposium will help to disseminate among medical men and research workers some of the interesting findings in the field of lipid metabolism, including the abnormalities leading to atherosclerosis and its dreaded sequelae.

On behalf of the Foundation, I take this opportunity to express my grateful appreciation to Dr. Hartroft who graciously undertook to prepare the agenda for the symposium and act as its chairman, as well as moderator of the morning session, and to Dr. Artom, who, although recuperating from a long illness, made the trip to Philadelphia to moderate the afternoon session and join in the discussions.

I also wish to express the thanks of the Foundation to Drs. Lucas and Wilgram of Canada, and to Drs. Chow, Rodbard and Olson, among the invited guests who took part in the discussions. I also take this occasion to thank the respective grantees of the Foundation.

LOUIS FREEDMAN, PH.D.
Chairman, Executive Committee
Lipotropic Research Foundation
New York, New York

Introduction

LIPOTROPIC agents surely have a relationship to serum lipids. Dr. Cornatzer deals with that subject in his paper on lipid transport. One of the lipids that has received a great deal of attention is cholesterol. Dr. Lucas covers that subject. In the light of new knowledge of the last two or three years about cholesterol and its transport, the earlier work of Dr. Lucas and Dr. Ridout needs review. Dr. Friedman's work on the phosphatides is also very exciting. Some of you know about it; it is opening up a whole new field.

The pathologist has looked at serum lipids for years in frozen sections stained for fat of kidneys, livers and other organs and has recognized it by its sudanophilia. When the plasma becomes coagulated in the fixed tissue sections if there is very much lipid present, one sees it as a red matrix in an oil-red stained section. Sometimes it will look homogeneous and at other times granular.

Lipids can also be visualized with the electron microscope. In a capillary in the liver the lipids appear as little black dots. They lack any demonstrable structure and one cannot see any membranes around them or an internal light area that might represent protein. In sections prepared from rats on a choline-deficient diet, the droplets do not seem as numerous as in those from rats fed a choline-supplemented diet. But, of course, this method is not quantitative. In order to make it so, hundreds of sections would have to be cut and measurements carried out. This might be worthwhile later on if we are looking for special effects, but at the moment we do not propose to go any further with this. We now look to the biochemist and enzymologist for leadership in this area.

W. STANLEY HARTROFT, M.D., PH.D.
Chairman, Department of Pathology
School of Medicine
Washington University
St. Louis, Missouri

Mechanism of Action of Choline

CAMILLO ARTOM, M.D.*

IT IS obvious that an accumulation of fat such as is found in the liver of animals with choline deficiency may result from a change in the rate of a variety of metabolic processes. For instance, it may be due to an increased synthesis, a decreased oxidation in the liver, an increased mobilization from the depots to the liver or a decreased mobilization from the liver to other tissues. Accordingly, the lipotropic action of choline could be explained by a stimulation or inhibition of any one or more of such processes.

During the last twenty years a number of investigators have attempted to choose between these various interpretations by giving heavy water,[1–4] or, more recently, C^{14}-acetate[4,6] to animals with or without a deficiency of choline, and then comparing the amounts and concentrations of the newly synthesized fatty acids in the livers and depots of these two groups of animals. The results of recent experiments carried out in our laboratory on mice fed labeled acetate[6] are essentially in agreement with those previously obtained by others. In all animals the specific activities of the fatty acids were higher in the liver than in the muscle, suggesting that the labeled fatty acids which accumulate in the liver are chiefly synthesized in this tissue rather than originating in the depots. On the other hand, no significant differences were found when the specific activities of the fatty acids in the animals receiving, or not receiving, choline were compared. This might be taken as an indication that choline does not affect either the synthesis or the oxidation of fatty acids.

However, a simple calculation of the differences in the fatty acid content of the choline-deficient and non-choline-deficient livers shows that such differences could well be explained by small changes in the rate of either the synthesis or the disposal of fatty acids, and that these changes might be small enough to fall within the limits of error of the isotopic determinations.

Because of the difficulty of interpreting the results obtained on intact animals, we thought years ago that more direct information on a possible role of choline in the oxidation of fatty acids could be obtained in experiments on isolated preparations of the liver. In this type of experiment, the use of labeled fatty acids as substrates seemed quite appropriate, since, by determining the isotopic concentration in the carbon dioxide and acetoacetate produced, changes in the oxidation rate of fatty acids could be detected independently from changes in the over-all metabolism of the tissue. Moreover, because of the sensitivity of radioactive measurements, extremely small amounts of isotopic substrates can be used, and thus one may minimize the well known inhibition of oxidation which occurs when fatty acids are added to tissue preparations.

In each of our experiments,[7] isolated preparations of the liver were incubated with C^{14}-stearate, or C^{14}-palmitate. The livers of choline-deficient rats produced much less isotopic carbon dioxide and acetoacetate than similar preparations from the livers of rats which had been maintained on the same diet, but which had received choline in the diet, or by injection shortly before sacrifice. Since similar differences were found with liver slices as well as with unfractionated homogenates, or mitochondria, such differences cannot be due to a change in the permeability of the cell membranes. Moreover,

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From the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina.

* Head, Department of Biochemistry.

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since most fat is eliminated in the preparation of the mitochondria, our results could not be ascribed to a greater isotopic dilution of the substrate by the neutral fat accumulating in the choline-deficient livers.

The last statement is further corroborated by the results of more recent experiments[6] in which we have studied the distribution of the isotopic carbon in the neutral fat, phospholipid and free fatty acid fractions of the incubated livers. During the incubation, part of the isotopic fatty acid is incorporated into the neutral fat and phospholipid, the amounts incorporated being almost identical in the deficient or non-deficient livers. The only differences between these two types of livers are in the C^{14} contents of the free fatty acids remaining at the end of incubation. These correspond almost exactly to the opposite differences in the $C^{14}O_2$ and C^{14}-acetoacetate produced during the incubation. In other words, it seems that the oxidation has occurred at the expense of the free fatty acid only, and that an isotopic dilution of this fraction by the neutral fat present in the tissue is unlikely.

In subsequent experiments with a similar technic,[8] we have shown that, besides the liver, other tissues of the deficient animals, especially the kidney and heart, exhibit a decreased ability to oxidize fatty acids, and that this decrease is also prevented by the administration of choline to the animals. These findings may have some interest in relation to the problem of the development of atherosclerotic lesions in the myocardium and kidney of choline-deficient animals.

While oxidation of fatty acids in the deficient livers was enhanced by administration of choline *in vivo*, addition *in vitro* of choline or simple choline derivatives, such as betaine aldehyde, betaine or phosphorylcholine, was totally ineffective. These results suggest that the active factor is not free choline, but some compound which is formed from choline *in vivo*, not *in vitro*, and which is firmly retained in the granular structures of the cell, even after repeated washings as in the preparation of mitochondria. The most obvious possibility was that choline enhanced oxidation of fatty acids by promoting the formation of tissue lecithins.

Actually, a comparative analysis of the data obtained from the animals receiving, or not receiving, choline indicated a rough parallelism between the level of lecithins in the liver and the ability of the isolated tissue to oxidize fatty acids added *in vitro*. Furthermore, when a single large dose of choline was injected into choline-deficient animals and these animals were killed at various intervals up to three hours after injection, both the level of lecithin and the rate of oxidation of fatty acids *in vitro* increased progressively.[6]

In several other series of experiments,[6,9] we also investigated whether or not, after administration of substances which directly or indirectly might interfere with the synthesis of choline or of choline-containing phospholipids, the amounts of lecithins and the ability of the liver to oxidize fatty acids would show changes in the same direction. This was the case when the diet was supplemented with triethylcholine, the effects of this compound being similar to those observed when choline was given. However, administration of diethanolamine, an analogue of ethanolamine, was followed by a decrease in the liver lecithins, whereas the isolated tissue still oxidized fatty acids at a high speed. After rats were given ethionine, a methionine analogue, the *in vitro* oxidation of fatty acids was greatly inhibited, whereas lecithin levels were as high as in the control animals. It seems, therefore, that a correlation between the levels of lecithins in the liver and the ability of the tissue to oxidize fatty acids is apparent in some but not in all conditions, and that livers with a low content of lecithins, as in the animals fed diethanolamine, are still quite competent to carry out the *in vitro* oxidation of fatty acids.

Recent observations from this and other laboratories have introduced some further complications in the interpretation of the results of our earlier experiments. The low casein diets which we used in our experiments were deficient not only in choline, but also in sulfur-containing amino acids. When we supplemented these diets with either methionine or cystine,[10] the oxidation of fatty acids in the isolated liver was raised to about the same degree as after supplementation with choline. Addition of tocopherol to the deficient diet was also effective. On the

other hand, when instead of a 5 or an 8 per cent diet, we used a diet containing 15 per cent of alpha protein from soybeans, the effects of choline administration on the oxidation of fatty acids *in vitro* became less marked and less consistent. Similar results have recently been published by other investigators who also used relatively high levels of proteins with a very low content of methionine.[4,11] It would seem that a considerable decrease in the ability of the isolated liver to oxidize fatty acids occurs only when the choline deficiency is combined with a severe protein deficiency. Such a decrease can be prevented to a large extent by supplementing the diet with choline, cystine or tocopherol, or just by raising the protein content of the diet without substantially increasing the supply of methionine or cystine.

CONCLUSIONS

The present evidence for a role of choline in the oxidation of fatty acids remains restricted to the results of *in vitro* experiments on the liver of animals previously maintained on low protein diets. We do not know whether or not this evidence can be extended to the conditions of the intact animal. The lipotropic action of choline probably cannot be explained only by an increased oxidation of fatty acids in the liver, and therefore the hypothesis that choline stimulates the mobilization of fatty acids from the liver is still a likely possibility. Actually, the two interpretations are not mutually exclusive. For instance, one might postulate that a deficiency of choline impairs the formation of liver lipoproteins, containing lecithins as their lipid moiety. These lipoproteins could be a transport form for fatty acids in blood plasma, and could also be important for the functional integrity of the mitochondria of the liver, perhaps by maintaining the spatial configuration of the enzymes involved in the oxidation of fatty acids.

Aside from any speculations, it is obvious that an explanation of the mechanism of the action of choline will not be fully acceptable until we can express this mechanism in chemical terms and at the molecular level. For the moment we have to confess that very little is known about this topic, and I can only express the hope that, by finding a newer approach and more appropriate tools, some younger investigators will be finally able to solve this interesting problem.

REFERENCES

1. Barrett, H. M., Best, C. H. and Ridout, J. H. Study of source of liver fat using deuterium as indicator. *J. Physiol.*, 93: 367, 1938.
2. Stetten, D., Jr. and Grail, G. F. The rates of replacement of depot and liver fatty acids in mice. *J. Biol. Chem.*, 148: 509, 1943.
3. Stetten, D., Jr. and Salcedo, J., Jr. The source of the extra liver fat in various types of fatty livers. *J. Biol. Chem.*, 156: 27, 1944.
4. (*a*) Bernhard, K., Ulbrecht, G., Ulbrecht, M. and Wagner, H. Versuche zur Erfassung der Fett und Glycogensynthese in der Leber cholinefrei ernährter Ratten mit Hilfe von C^{14}-Acetate und D-signierten Glycerin. *Helvet. chim. acta*, 37: 1439, 1954.
   (*b*) Bernhard, K., Ulbrecht, G., Ulbrecht, M. and Wagner, H. Alimentär bedingte Leberverfettung und extrahepatogene Fettbildung. *Helvet. physiol. et pharmacol. acta*, 14: 342, 1956.
5. Guggenheim, K. and Olson, R. E. Studies of lipogenesis in certain B-vitamin deficiencies. *J. Nutrition*, 48: 345, 1952.
6. Artom, C. Role of choline in the hepatic oxidation of fat. *Am. J. Clin. Nutrition*, 6: 221, 1958.
7. Artom, C. Role of choline in the oxidation of fatty acids by the liver. *J. Biol. Chem.*, 205: 101, 1953.
8. Artom, C. Effect of choline administration on the oxidation of fatty acids by extrahepatic tissues. *J. Biol. Chem.*, 213: 681, 1955.
9. Artom, C. Lipide choline and fatty acid oxidation in rat liver preparations. *Fed. Proc.*, 15: 690, 1956.
10. Artom, C. Effects of some dietary factors on the metabolism of fatty acids in liver preparations. *J. Biol. Chem.*, 223: 389, 1956.
11. Fritz, I. B. Effects of choline deficiency and carnitine on palmitic acid oxidation by rat liver homogenates. *Am. J. Physiol.*, 190: 449, 1957.

Lipotropic Agents and Lipid Transport

W. E. CORNATZER, M.D., PH.D.*

THE term "lipotropic" was first used by Best[1] to describe the action of choline in the prevention and cure of fatty liver. Historically, this term originated from the addition of egg yolk lecithin to the diet of depancreatized dogs in an attempt to prevent the formation of a fatty liver.[2] The identification of choline as the active constituent of the lecithin molecule was established the same year by these investigators.[3] It is of interest that the lipotropic action of choline not only involves the liver, but also other tissues such as the kidney,[4] aorta and blood vessels.[5,6]

The first report of the effects of low protein diet in the production of a fatty liver was reported by Best in 1932.[1,7,8] Tucker and Eckstein showed that the lipotropic constituent of protein was methionine.[9] The role of other amino acids, such as threonine and lysine, in the lipotropic action of methionine has also been investigated.[10] Betaine was reported in 1932 to be an effective lipotropic agent.[4] Inositol,[11] vitamin B_{12} and folic acid,[12] and manganese[13] under certain conditions are active lipotropic agents.

The materials which are physiologically active in removing fat from the liver were shown to be lecithins and choline-containing phospholipids, whereas the cephalins containing ethanolamine were inactive.[4] The composition of liver lipids can be altered by the dietary intake of protein. There is a decrease in the level of total phospholipid in the liver of animals maintained on a low protein diet. This decrease is especially marked in the lecithin fraction.[14] The liver is the main source of phospholipids in the plasma. Plasma phospholipids show a direct reflection of turnover in the liver of the rat,[15] dog[16] and man.[17] The phospholipids of the plasma are primarily choline-containing (70 to 80 per cent).[18] A choline deficiency results in a 30 to 40 per cent decrease in the plasma phospholipids.[19] The synthesis of lecithins in the liver of animals maintained on a low protein diet is approximately the same as in rats maintained on a stock diet,[20] but when guanidoacetic acid[21] or diethanolamine[22] is supplemented in the diet, a marked decrease occurs in the synthesis of lecithins.

Phospholipid synthesis in the liver is a primitive function, and much stress has to be applied if there are to be alterations in this function. The phospholipid synthesis in the plasma of untreated cirrhotic patients[23] or patients with infectious hepatitis[24] is similar to that found in normal persons. The synthesis of liver phospholipid in animals maintained on various diets is constant when expressed as per gram of fat-free tissue,[25] or remains unaltered in the production of dietary cirrhosis. Lipid phosphorylation is greatest in conditions in which a single dose of lipotropic agents is administered in the presence of a fatty liver.[26]

The effect of a dose of choline is chiefly due to the increased rate of formation of lecithins. The stimulating effect of various incomplete methylated ethanolamine derivatives was greatest in lecithin fractions of the liver in rats maintained on a low protein diet.[27] In cirrhotic patients with fatty infiltration in the liver, a significant increase in the rate of phospholipid synthesis was demonstrated by biopsy, after a single oral dose of choline or intravenous administration of methionine.[28] This stimulating effect of lipid phosphorylation by lipotropic agents is somewhat related to the previous diet, for it does not occur

From the Department of Biochemistry, University of North Dakota, Grand Forks, North Dakota.

* Head, Department of Biochemistry.

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in experimental animals on a stock diet[29] or in man on an adequate protein intake.[30]

The lipotropic action of choline is primarily involved in the mitochondria and large granules in the liver cell. A decrease occurred in the total lipid and lecithin phospholipids in the liver mitochondria of those animals maintained on a 5 per cent casein diet supplemented with 1 per cent guanidoacetic acid for two weeks.[31] The administration of a single dose of choline (40, 75 or 150 mg.) six hours before sacrificing the animals maintained on a choline-deficient diet produced a significant increase in lecithin phospholipid of the mitochondria. This effect of a single dose of choline (150 mg.) was demonstrated within three, six or ten hours following administration.[32] This observation and that of Artom[33] indicate that mitochondria are primarily involved in the lipotropic mechanism of choline. Lipid analyis of cellular fractions in liver necrosis has demonstrated a decrease in lecithin phosphorus in nuclei.[34]

The lipotropic and lipid phosphorylating effects of choline, betaine and inositol were compared in the liver of rats maintained on low protein-low fat or low protein-high fat diets.[35] All three agents were equally effective in causing a significant reduction of total lipids over controls in animals maintained on the low protein-low fat diet. In rats maintained on the high fat diet, only choline and betaine supplements caused a substantial reduction of total liver fat. Phospholipid synthesis was increased when choline, betaine or inositol was administered as a single dose to animals maintained on the low protein-low fat diet. The order of effectiveness upon turnover rate was choline > betaine > inositol. Both choline and betaine, when administered as single doses, stimulated lipid phosphorylation in rats on the high fat diet, but inositol did not.[35,36] This lipotropic response of choline and betaine was similar to that observed by Young et al.[37] and Morrison.[38] Small doses of methionine are not lipotropic: however, a lipotropic effect was observed in those animals to which threonine was incorporated in the diet along with methionine.[39] It was postulated that methionine stimulates protein metabolism in animals maintained on a low protein diet supplemented with small amounts of this amino acid and therefore methionine fails to exert its lipotropic effect. In order to evaluate this conclusion the uptake of S^{35}-methionine into the sulfur-containing amino acids in liver proteins was studied in rats maintained on diets with and without choline. The evidence presented supports the hypothesis that protein metabolism takes precedence over lipotropic action when animals are subjected to a dietary stress of methyl group deficiency.[40]

Considerable progress has been made in possible pathways of phospholipid synthesis in the liver for partial purified enzyme systems.[41] The *in vivo* effect of coenzyme A, adenosine triphosphate, adenosine-5′-phosphate, cytidine-5′-phosphate and oxalacetate on lipid phosphorylation in the liver of rats has been investigated in choline-deficient rats.[42] A single dose of coenzyme A stimulates phospholipid synthesis in choline-deficient and in pantothenic-deficient animals; oxalacetate stimulates in choline-deficient animals. Administration of a single dose of a mixture of coenzyme A, adenosine-5′-phosphate, oxalacetate and choline stimulates lipid phosphorylation in both mitochondria and nuclei greater than choline. The greatest stimulation occurred in the lecithin fraction. There is considerable evidence that the sulfur-containing amino acids are involved in the production of liver necrosis induced by chemical as well as dietary means. A pronounced decrease in the free sulfhydryl compounds in the liver preceded the production of necrosis. The decreased sulfhydryl concentration occurred in the liver and kidney and to a lesser extent in the heart and spleen.[43]

In order to discuss lipid transport it is necessary to mention the origin and fate of plasma lipids, chemistry of β-lipoproteins, mechanism of hyperlipemia and absorption of lipids. I have already mentioned some facts about the origin and fate of plasma phospholipids. The absolute turnover of phospholipids in man has been reported by Emerson as 18 gm. per day whereas the turnover of cholesterol has been found to be only 1 to 3 gm. Thus, the liver plays a predominant

role in the production and utilization of plasma phospholipids and cholesterol.

SUMMARY

The lipotropic action of choline, betaine, inositol, methionine and dietary proteins is discussed.

The lipotropic action of choline shows its effect not only in the liver but also in other tissues, such as kidney, aorta and other blood vessels. Phospholipids containing choline are involved in this lipotropic process, effecting primarily the mitochondria and large granules in the liver cell.

The effect of a single dose of lipotropic agent is chiefly due to the increased rate of formation of lecithins. In cirrhotic patients with fatty infiltration in the liver, a significant increase in the rate of phospholipid synthesis was demonstrated by biopsy after a single dose of choline or intravenous administration of methionine. This stimulating effect of lipid phosphorylation by lipotropic agents does not occur in experimental animals maintained on stock diet or in man on an adequate protein intake.

REFERENCES

1. Best, C. H., Hershey, J. M. and Huntsman, M. E. The effect of lecithin on fat deposition in the liver of the normal rat. *J. Physiol.*, 75: 56, 1932.
2. Hershey, J. M. and Soskin, S. Substitution of "lecithin" for raw pancreas in the diet of the depancreatized dog. *Am. J. Physiol.*, 98: 74, 1931.
3. (*a*) Best, C. H., Hershey, J. M., and Huntsman, M. E. The control of the deposition of liver fat. *Am. J. Physiol.*, 101: 7, 1932.
   (*b*) Huntsman, M. E. The effects of the components of lecithine upon deposition of fat in the liver. *J. Physiol.*, 75: 405, 1932.
4. Griffith, W. H. and Wade, N. J. I. The occurrence and prevention of hemorrhagic degeneration in young rats on a low choline diet. *J. Biol. Chem.*, 131: 567, 1939.
5. Kesten, H. D., Salecdo, J., Jr. and Stetten, D., Jr. Fatal myocarditis in choline deficient rats fed ethyl laurate. *J. Nutrition*, 29: 171, 1945.
6. Hartroft, W. S., Ridout, J. H., Sellers, E. A. and Best, C. H. Atheromatous changes in aorta, carotid and coronary arteries of choline-deficient rats. *Proc. Soc. Exper. Biol. & Med.*, 81: 384, 1952.
7. Best, C. H. and Huntsman, M. E. The effect of choline on the liver fat of rats in various states of nutrition. *J. Physiol.*, 83: 255, 1935.
8. Best, C. H., Huntsman, M. E. and Ridout, J. H. The "lipotropic" effect of protein. *Nature, London*, 135: 821, 1935.
9. Tucker, H. F. and Eckstein, H. C. The effect of supplementary methionine and cystine on the production of fatty livers by diet. *J. Biol. Chem.*, 121: 479, 1937.
10. (*a*) Singal, S. A., Hazan, S. J., Sydenstricker, V. P. and Littlejohn, J. M. The production of fatty livers in rats on threonine- and lysine-deficient diets. *J. Biol. Chem.*, 200: 867, 1953.
    (*b*) Harper, A. E., Monson, W. J., Benton, D. A. and Elvehjem, C. A. The influence of protein and certain amino acids, particularly threonine, on the deposition of fat in the liver of the rat. *J. Nutrition*, 50: 383, 1953.
11. Gavin, G. and McHenry, E. W. Inositol: a lipotropic factor. *J. Biol. Chem.*, 139: 485, 1941.
12. (*a*) György, P. and Goldblatt, H. Treatment of experimental dietary cirrhosis of the liver in rats. *J. Exper. Med.*, 90: 73, 1949.
    (*b*) Hall, C. A. and Drill, V. A. Lipotropic effect of liver extract on dietary hepatic injury in rats. *Proc. Soc. Exper. Biol. & Med.*, 69: 3, 1948.
    (*c*) Drill, V. A. and McCormick, H. M. Lipotropic effects of vitamin B_{12} concentrate. *Proc. Soc. Exper. Biol. & Med.*, 72: 388, 1949.
13. Admur, M., Norris, L. C. and Heuser, G. F. The lipotropic action of manganese. *J. Biol. Chem.*, 164: 783, 1946.
14. (*a*) Artom, C. and Fishman, W. H. The relation of the diet to the composition of tissue phospholipids. II. Changes in tissue phospholipids induced by experimental diets. *J. Biol. Chem.*, 148: 415, 1943.
    (*b*) Fishman, W. H. and Artom, C. The relation of the diet to the composition of tissue phospholipids. IV. The action of choline and choline precursors in weanling rats. *J. Biol. Chem.*, 154: 109, 1944.
    (*c*) Fishman, W. H. and Artom, C. The relation of the diet to the composition of tissue phospholipides. VI. Liver lecithin as related to the choline and fat content of the diet. *J. Biol. Chem.*, 164: 307, 1946.
15. Zilversmit, D. B. and Bollman, J. L. Role of the liver and intestine in the turnover of plasma phosphatides in the rat. *Arch. Biochem.*, 63: 64, 1956.
16. Chaikoff, I. L. and Zilversmit, D. B. Radioactive phosphorus: its application to the study of phospholipid metabolism. *Advances Biol. & Med. Phys.*, 1: 321, 1948.
17. Cornatzer, W. E. Phospholipid metabolism in human liver disease. In: Phosphorus Metabolism, vol. 2, p. 243. Edited by McElroy, W. D. and Glass, B. Baltimore, 1952. Johns Hopkins Press.

18. ALBRINK, M. J. The choline-containing phospholipids of serum. *J. Clin. Invest.*, 29: 46, 1950.
19. ROSENFELD, B. and LANG, J. M. Influence of dietary choline and protein on the level of phospholipid in the serum of the rat. *Canad. J. Biochem. & Physiol.*, 35: 845, 1957.
20. ARTOM, C. and CORNATZER, W. E. Lipide phosphorylation in the liver as related to the dietary supply of methyl donors and methyl acceptors. *J. Elisha Mitchell Sc. Soc.*, 65: 190, 1949.
21. (*a*) ARTOM, C. and CORNATZER, W. E. Lipide phosphorylation in the liver as related to the dietary supply of methyl donors and methyl acceptors. Procedure of the First International Congress of Biochemistry. Cambridge, England, p. 22, 1949.
(*b*) ARTOM, C. Formation of phospholipides in animal tissue. In: Phosphorus Metabolism, vol. 2, p. 203. Edited by McElroy, W. D. and Glass, B. Baltimore, 1952. Johns Hopkins Press.
22. ARTOM, C., CORNATZER, W. E. and CROWDER, M. The action of an analogue of ethanolamine (diethanolamine) on the formation of liver phospholipides. *J. Biol. Chem.*, 180: 495, 1949.
23. CORNATZER, W. E. and CAYER, D. The effects of lipotropic factors on phospholipide turnover in the plasma of patients with cirrhosis of the liver as indicated by radioactive phosphorus. *J. Clin. Invest.*, 29: 542, 1950.
24. CAYER, D. and CORNATZER, W. E. The effects of lipotropic factors on the phospholipide turnover in patients with infectious hepatitis. *Gastroenterology*, 18: 79, 1951.
25. (*a*) CORNATZER, W. E. and ARTOM, C. Unpublished data.
(*b*) ARTOM, C. Mechanism of action of lipotropic factors in animals. Tenth Conference on Liver Injury, p. 62. New York, 1951. Josiah Macy, Jr. Foundation.
26. CAYER, D. and CORNATZER, W. E. The use of lipotropic factors in the treatment of liver disease. *Gastroenterology*, 20: 385, 1952.
27. ARTOM, C., and CORNATZER, W. E. The action of ethanolamine, methylethanolamine, and dimethylethanolamine on lipide phosphorylation. *J. Biol. Chem.*, 176: 949, 1948.
28. WILLIAMS, J., CAYER, D. and CORNATZER, W. E. Lipotropic factors in the treatment of cirrhosis. *South. M. J.*, 44: 369, 1951.
29. ARTOM, C. and CORNATZER, W. E. The action of choline and fat on lipide phosphorylation in the liver. *J. Biol. Chem.*, 171: 779, 1947.
30. CORNATZER, W. E. and CAYER, D. The effects of lipotropic factors on phospholipide turnover in the plasma of normal persons as indicated by radioactive phosphorus. *J. Clin. Invest.*, 29: 534, 1950.
31. GALLO, D. G. Thesis: Phospholipide metabolism in cellular fractions. University of North Dakota, Grand Forks, 1955.
32. CORNATZER, W. E. and GALLO, D. G. Mitochondrial phospholipides in cirrhosis. *Am. J. Med.*, 21: 136, 1956.
33. ARTOM, C. Role of choline in the oxidation of fatty acids by the liver. *J. Biol. Chem.*, 205: 101, 1953.
34. CORNATZER, W. E. and GALLO, D. G. Lipide analysis of cellular fractions in liver necrosis. *Proc. Soc. Exper. Biol. & Med.*, 93: 287, 1956.
35. CORNATZER, W. E. and SIMONSON, G. E. A lipotropic and lipide phosphorylation comparative study of choline, betaine and inositol. Abstract of the American Chemical Society 126th Meeting, p. 54C. New York, 1954.
36. SNYDER, F., CORNATZER, W. E. and SIMONSON, G. E. Comparative lipotropic and lipide phosphorylating effects of choline, betaine, and inositol. *Proc. Soc. Exper. Biol. & Med.*, 96: 670, 1957.
37. YOUNG, R. J., LUCAS, C. C., PATTERSON, J. M. and BEST, C. H. Lipotropic dose-response studies in rats: comparisons of choline, betaine, and methionine. *Canad. J. Biochem. & Physiol.*, 34: 713, 1956.
38. MORRISON, L. M. Use of betaine-lipotropic combinations in clinical practice. *Geriatrics*, 8: 649, 1953.
39. CORNATZER, W. E. and SNYDER, F. The role of threonine on the lipotropic action of methionine. *Proc. North Dakota Acad. Sc.*, 9: 15, 1955.
40. SNYDER, F. and CORNATZER, W. E. Effect of choline on the incorporation of S^{35}-L-methionine into liver protein of rats with fatty livers. *Biochim. et biophys. acta*, 27: 650, 1958.
41. (*a*) KENNEDY, E. P. and WEISS, S. B. Cytidine diphosphate choline: a new intermediate in lecithin biosynthesis. *J. Am. Chem. Soc.*, 77: 250, 1955.
(*b*) KENNEDY, E. P. and WEISS, S. B. The function of cytidine coenzymes in the biosynthesis of phospholipides. *J. Biol. Chem.*, 222: 193, 1956.
42. (*a*) CORNATZER, W. E. and SNYDER, F. *In vivo* effect of coenzyme A on lipide phosphorylation. *Fed. Proc.*, 14: 197, 1955.
(*b*) CORNATZER, W. E. and SNYDER, F. *In vivo* effect of CoA and other factors on lipide phosphorylation. *Proc. Soc. Exper. Biol. & Med.*, 94: 363, 1957.
43. SNYDER, F. and CORNATZER, W. E. Sulfur metabolism in chemically induced necrotic liver degeneration. *J. Biol. Chem.*, 231: 839, 1958.

Lipid Transport

H. C. TIDWELL, PH.D.*

THE first mechanism in the transport of lipids necessarily involves the passage of the lipid across the intestinal barrier. A better understanding of the exact nature of the forms required for intestinal absorption of glycerides should give further insight into their transport across all membranes in the body.

TRANSPORT ACROSS THE MUCOSA

The generally accepted theory of the mechanism of fat absorption has varied markedly during the last few years. The "partition hypothesis" of Frazer[1] gained acceptance for a time. According to this hypothesis, most of the triglycerides are absorbed as such, then pass through the chyle into the systemic circulation, while a smaller, hydrolyzed fraction goes directly into the portal blood. It now appears to be well established by Bloom et al.[2] that most if not all of the absorbed lipid enters the circulation by way of the lymphatics.[3] Reiser[4] and Dowse[5] and their associates have reported that only trace amounts of triglyceride are absorbed as such. Numerous investigators have suggested that up to 50 per cent of the ingested fat is completely hydrolyzed before absorption. A partial hydrolysis of the remainder of the triglyceride would necessitate the absorption of at least two-thirds of the ingested glyceride as free fatty acids.

Recent studies from our laboratory[6] support the hypothesis that most of the fatty acids of triglycerides are absorbed, not as glycerides, but as unesterified fatty acids. Evidence for this conclusion can be summarized in four categories:

From the Department of Biochemistry, University of Texas Southwestern Medical School, Dallas, Texas.

* Professor and Chairman.

Presented at the First Symposium of the Lipotropic Research Foundation, April 13, 1958, Philadelphia, Pennsylvania.

(1) Ingested fatty acids are at least as rapidly absorbed as fed triglycerides if only the amounts actually available for absorption are considered. In support of this, Borgström[7] contends that fatty acids are selectively absorbed when small amounts are fed with a fat.

(2) The percentage of fatty acids in the intestine during fat absorption bears a closer relationship to the rate of lipid absorption than does the degree of emulsification.

(3) The rate of fat absorption was not promoted by the presence of the mixture of fatty acids, monoglycerides and triglycerides, which Frazer found to be ideal for promoting emulsification.

(4) Fat supplements, which accelerate the action of the lipolytic enzymes, are associated with marked increases in the rate of absorption of an ingested fat. Supplements such as histidine and ethionine increased the rate of enzymatic hydrolysis and absorption, while glycine and methionine did not.[6] Hence any effect of methionine on absorption cannot be the result of an increased rate of hydrolysis of the fat.

Whether phospholipids play an important role in the absorption and conversion of fatty acids to glycerides is not known. Studies on phosphorous turnover in the intestinal wall have indicated that the phospholipids cannot have a major role in the absorption of fats.[8] This may not be true if only one form of phospholipid is actively involved, or if phosphoryl choline should be transferred intact instead of breaking down to phosphate and choline. Nevertheless, choline and possibly methionine were found to hasten the absorptive process and this result might be associated with an increased formation of phospholipid. This has been questioned by others when results were obtained under conditions different from ours. How-

ever, phospholipids may have some part in the absorption and transport of fatty acids across the intestinal barrier. Eventually, the fatty acids enter the chyle and appear in the blood as chylomicrons, consisting principally of triglycerides in association with small amounts of phospholipid, cholesterol and protein.

TRANSPORT IN THE BLOOD

Two different theories have been advanced regarding the mechanism of transport of neutral fat in the blood. One group of investigators believes that the β-lipoproteins are involved in the transport of the fatty acids derived from the chylomicrons of the blood, while another group assigns the major role in this process to albumin.

Korn[9] has produced evidence that the triglycerides in the chyle may be associated with small amounts of protein (probably β-lipoprotein) as chylomicrons. The triglycerides of the chylomicrons and the low density β-lipoproteins may be acted upon by lipoprotein lipase to yield free fatty acids and possibly high density β-lipoproteins. This process frees the lipoproteins which then can form more complexes with fat. Hence, the lipoproteins may serve as intermediates in the conversion of the triglycerides of the chylomicrons into complexes which can be acted upon by lipoprotein lipase.

Albrink et al.[10] have found the major part of the lipid in the blood after a fat meal in the chylomicrons, which are largely neutral fat with small amounts of phospholipid and cholesterol. During a fasting period, most of the lipid is in soluble form having a much higher proportion of cholesterol and phospholipid than neutral fat. These investigators have presented evidence to support the thesis that the labile, rapidly removable, neutral fat is transported by the more stable lipoprotein complex, which consists basically of cholesterol, phospholipid and protein. Swank and Fellman,[11] have found that an injection of a sizable amount of chyle is followed by a large increase in plasma β-globulins, and later by increases in α-globulins. This supports the aforementioned theory. Similarly, Jones et al.[12] have presented evidence to suggest that low density lipids are involved in the transport of fatty acids.

An opposing theory is favored by Bergström,[13] Havel[14] and their co-workers. Earlier, Bergström[13] had found a rapid turnover of absorbed fatty acids, as much as 1 mg. per minute over a four-hour period, even when the absorbed labeled lipid amounted to a maximum of only 15 per cent of the total plasma lipids. In other words, the absorbed triglycerides were not mixed with the existing plasma lipids, which were mainly in lipoprotein combinations. Havel et al.[14] found 97 per cent of the absorbed activity from ingested labeled fat in the triglyceride of the chylomicrons in the chyle, which contained little if any unesterified fatty acid. After injecting labeled chylomicrons, little activity was found in the α- or β-lipoproteins. The percentage of the unesterified fatty acid increased as the chylomicron content decreased. The unesterified fatty acids had a half-life of about two minutes, while that of the chylomicrons was about twenty minutes. Their data do not support the concept that other lipoproteins are involved in the removal of the lipid of the chylomicrons from the plasma. The site of lipolysis of the triglyceride is not known.

Rodbell[15] has recently obtained additional evidence that, starting with chylomicrons, lipoproteins rich in triglycerides are not gradually transformed from lower into higher density lipoproteins by the successive removal of triglyceride. If the transformation were due only to loss of lipid, the protein would be the same in both low and high density lipoproteins. He found that the various types of lipoproteins did not contain a common protein as judged by content of some amino acids. However, this would not exclude the possibility that the unesterified fatty acid may combine with certain of the blood protein fractions for fatty acid transport.

We found that the activity associated with the super layer, after centrifuging out the chylomicrons, had increased from about 30 to 60 per cent of the total blood activity at five hours as compared with one and a half hours after feeding a labeled fat.[16] A trichloroacetic acid precipitation of the proteins revealed that

only about half of the activity of the precipitate could be extracted with fat solvents even after treatment with alkali. At this longer interval, a considerable part of the remaining activity was associated with a protein fraction whose nature is yet to be determined.

Dole,[17] Gordon[18] and co-workers suggested that the metabolically active form of fat in the body is the unesterified fatty acids. It also may be true that this is the major form involved in the transfer of most of the fat across all cell membranes.[19]

The exact role of lecithin in lipid transport is as yet unspecified and that of cholesterol is still to be determined. If the lipoproteins do aid in the transport, it is possible that the fundamental role of these two lipids may be associated with structure.

Much of the conflicting evidence reported can probably be explained by the use of different kinds of experimental animals under quite different experimental conditions. Many of these studies reported were made and conclusions drawn without due regard to species differences which now appears quite evident in some cases.

SUMMARY

Available evidence suggests that most of the fatty acids of ingested triglycerides are absorbed as unesterified fatty acids and not as glycerides. Some phospholipid fraction may be involved in the transport of the fatty acids into the mucosa or their conversion into triglycerides. After release into the blood largely as triglycerides in chylomicron form, the fatty acids are transported at least partly with the aid of the blood lipoproteins. It is possible that the unesterified fatty acids released from these lipoproteins are the most metabolically active form of fat and that they are the major form involved in the transport of fat across cell membranes.

REFERENCES

1. Frazer, A. C. The absorption of triglyceride fat from the intestine. *Physiol. Rev.*, 26: 103, 1946.
2. Bloom, B., Chaikoff, I. L., Reinhardt, W. O., Entenman, C. and Dauben, W. G. Quantitative significance of lymphatic pathway in transport of absorbed fatty acids. *J. Biol. Chem.*, 184: 1, 1950.
3. Borgström, B. On the mechanism of the intestinal fat absorption. *Acta chem. scandinav.*, 5: 643, 1951.
4. Reiser, R. and Diekert, J. W. Intestinal absorption of unhydrolyzed tripalmitin. *Proc. Soc. Exper. Biol. & Med.*, 92: 649, 1956.
5. Dowse, C. M., Saunders, J. A. and Schofield, B. The composition of lipid from jejunal contents of the dog after a fatty meal. *J. Physiol.*, 134: 515, 1956.
6. Tidwell, H. C. Absorption of free and combined fatty acids. *Proc. Soc. Exper. Biol. & Med.*, 98: 12, 1958.
7. Borgström, B. On mechanism of intestinal fat absorption; metabolism of lipids. *Acta physiol. scandinav.*, 25: 291, 1952.
8. Zilversmit, D. B., Chaikoff, I. L. and Entenman, C. Are phospholipids obligatory participants in fat transport across intestinal wall? *J. Biol. Chem.*, 172: 637, 1948.
9. Korn, E. D. Clearing factor, a heparin-activated lipoprotein lipase. I. Substrate specificity and activation of coconut oil. *J. Biol. Chem.*, 215: 15, 1955.
10. Albrink, M. J., Glenn, W. W. L., Peters, J. P. and Man, E. B. Transport of lipids in chyle. *J. Clin. Invest.*, 34: 1467, 1955.
11. Swank, R. L. and Fellman, J. H. Plasma proteins and fat transport in dogs. *Am. J. Physiol.*, 192: 318, 1958.
12. Jones, J. B., Gofman, J. W., Lindgren, F. T., Lyon, T. P., Graham, D. M., Strisower, B. and Nichols, A. V. Lipoproteins in atherosclerosis. *Am. J. Med.*, 11: 358, 1951.
13. Bergström, S., Borgström, B. and Rottenberg, M. Intestinal absorption and distribution of fatty acids and glycerides in rat; metabolism of lipids. *Acta physiol. scandinav.*, 25: 120, 1952.
14. Havel, R. J. and Fredrickson, D. S. The metabolism of chylomicra. I. The removal of palmitic acid-1-C^{14} labeled chylomicra from dog plasma. *J. Clin. Invest.*, 35: 1025, 1956.
15. Rodbell, M. N-terminal amino acid and lipid composition of lipoproteins from chyle and plasma. *Science*, 127: 701, 1958.
16. Burr, W. W., Jr., McPherson, J. C. and Tidwell, H. C. Chylomicron metabolism: alterations of absorbed labeled lipid. *Am. J. Physiol.*, 195: 769, 1958.
17. Dole, V. P. The significance of nonesterified fatty acids in plasma. *Arch. Int. Med.*, 101: 1005, 1958.
18. Gordon, R. S., Jr. and Cherkes, A. Unesterified fatty acid in human blood plasma. II. The transport function of unesterified fatty acid. *J. Clin. Invest.*, 35: 206, 1956; 36: 810, 1957.
19. Fredrickson, D. S. and Gordon, R. S. Transport of fatty acids. *Physiol. Rev.*, 38: 585, 1958.

DISCUSSION

DR. W. STANLEY HARTROFT (*St. Louis, Missouri*): What has occurred in the last two or three years as a result of study with the electron microscope is the realization that the phenomenon of pinocytosis, which has been recognized for a long time and studied extensively in protozoa and amoeba, is apparently capable of being performed by all the cells of the body, even in mammals.

Using this method, the study of fats is going to be easy because the usual form of fixation for electron microscopy is osmium, which preserves the fat and makes it black.

We are becoming interested in the possibility that this phenomenon of pinocytosis may occur in the liver under normal conditions, and we wonder whether or not it is altered in choline deficiency. Our own investigations with the electron microscope are aimed in this direction but we have nothing to report at this state.

DR. TIDWELL: Dr. Frazer raised the question as to whether all fat has to be split into fatty acids before absorption. I do not think one can say with certainty that this is so, but some of our recent studies offer considerable evidence that would suggest that fatty acid is the form most likely to be absorbed by the intestine or to escape from the vascular bed.

For example, we found that despite general reports, the fatty acids were not more slowly absorbed than triglycerides. When fed as such, the fatty acids were retained in the stomach longer than triglycerides, as were the monoglycerides; whereas, if only the lipid in the intestine is considered as available for absorption, the fatty acids are removed just as rapidly, if not faster, than the triglycerides.

When the lipids were available in a form to best promote emulsification according to Frazer, there was no increase in the rate of absorption. We compared the effect of emulsification with that of an increase in the percentage of free fatty acids. There seemed to be a much closer relationship to the amount of free fatty acids than to the degree of emulsification. Feeding substances which promoted the rate of hydrolysis *in vitro* also markedly hastened the rate of absorption of the fat.

An investigator in our laboratory, who has been studying *in vitro* absorption, was unable to obtain any absorption into the mucosal cells of fat from emulsions of triglycerides under conditions at which hydrolysis could not readily take place. When a complex of albumin and free fatty acids were employed, he obtained a ready passage of these free fatty acids across the barrier. Undoubtedly, traces of glyceride may get into the cell as such, but available evidence suggests that the major part of the glycerides has to be split and enters in the form of free fatty acids.

DR. HARTROFT: Incidentally, Dr. Frazer described cilia as hollow, but this view has not been confirmed by electron microscopy.

DR. CAMILLO ARTOM (*Winston-Salem, North Carolina*): Dr. Tidwell, could absorption of fatty acids be to a large extent in the form of monoglycerides?

DR. TIDWELL: I do not think so. Of course, there is a possibility that limited amounts of all three glycerides may be absorbed. If at least half of the ingested triglyceride is completely hydrolyzed, at least two-thirds of the total amount of fatty acids will be present as free fatty acids. Undoubtedly, some of the glycerides do get across as such but the evidence is rather convincing that the major part of the lipid does not get across in the form of glycerides, either mono- or triglyceride.

DR. HARTROFT: I was glad that Dr. Cornatzer considered the nucleus in his studies. Some years ago Dr. Wilgram and I observed that stainable fat could be demonstrated in the hepatic nuclei of choline-deficient animals. It is not peculiar to the choline-deficient type of fatty liver, however. Recently, my associate, Dr. Grisham, has been measuring hepatic nucleus-nucleolar ratios and nuclear-cytoplasmic ratios in various stages of choline deficiency. He has found fat in the nuclei of these liver cells after less than two weeks of choline deficiency. Of course, we knew it appeared later when cirrhosis developed, but the fact that it occurred so early was startling.

Polyploidy also appeared at an early stage. We thought it might be a mechanical effect of an excess of fat in the cytoplasm but it also clearly occurs in cells having the least amount of fat.

Am I right in stating that you found phospholipid synthesis was stimulated in the nucleus?

DR. W. E. CORNATZER (*Grand Forks, North Dakota*): By giving a single dose of the mixture of CoA, CoA choline and AMP, greater than occurs in the nucleus and in the mitochondria, this effect was achieved.

DR. HARTROFT: But choline itself cannot produce this effect; other constituents must be added?

DR. CORNATZER: Yes. We have analyzed the lipid content in liver necrosis induced by bromobenzene and have found that there is a decrease in the lecithin phosphorus in the nucleus.

DR. HARTROFT: Is this in the necrotic nuclei or in the non-necrotic nuclei?

DR. CORNATZER: This is in the necrotic nuclei.

DR. HARTROFT: How do you separate them?

DR. CORNATZER: We separate them by a centrifugation method. The control subjects receive the corn oil and the subjects with necrosis receive corn oil plus the halogenated agent.

DR. HARTROFT: But in the animals that received the toxins, you have a mixture of necrotic and non-necrotic nuclei?

DR. CORNATZER: That is true. You are thinking of a dose of halogenated compound that Kutz, Weiser and Poplar used to induce massive necrosis. We have used that dose, and we probably still have some normal cells although I do not know the number. We have not separated them, but the dose used produces massive necrosis throughout the liver.

DR. G. WILGRAM (*Toronto, Canada*): I was

interested in the results obtained by Dr. Artom when the animals on a diet low in methionine were fed methionine and choline, and the specific activity of the labeled fatty acids was registered in the liver in the *in vitro* tests. When choline was added to this diet, it did not result in the complete removal of fat from the liver. However, we presume this would have occurred if large doses of methionine had been added to the diet, showing that although choline under conditions similar to yours has some effect, it does not compare with the effect achieved by administering large doses of methionine.

DR. CORNATZER: When choline is given by stomach tube, there is always some destruction of the choline by bacteria to trimethylamine. Once it reaches the liver cell there is still some destruction of the choline by choline oxidation *per se* as compared to methionine.

Methionine can go into active methionine, and Stetten has shown that the monomethyl group of choline comes only from methionine. The other methyl groups are not from methionine.

DR. WILGRAM: The point that I am considering is somewhat different. Does choline have the same effect as methionine on a low choline diet in increasing fatty acid oxidation? Dr. Artom's figures lead me to believe it has not.

DR. ARTOM: I believe the figures show no significant difference. Considering that the results are somewhat variable from one experiment to another, the two figures represent the same effect. I cannot see any difference.

DR. M. CHENOWETH (*Midland, Michigan*): I think that the combination of methionine and diethanolamine might be considered here. This combination would circumvent the excretion of choline, as some recent work on diethanolamine has shown.

DR. CORNATZER: Studies made some time ago by Dr. Artom and myself demonstrated the effect of diethanolamine as compared to choline. Diethanolamine on an equal molar basis stimulates more than choline, and the choline given by stomach tube is partially destroyed by choline oxidase, whereas choline oxidase has no effect on diethanolamine. Only one methyl group is required to convert diethanolamine to choline, and that methyl group, according to Stekol, apparently comes from methionine—available methionine in the liver cell, or active methionine.

DR. HARTROFT: Of course, if the choline was given in sufficient amounts, the effect should be obtained. Dr. Wilgram, did it matter how much choline you added?

DR. WILGRAM: No.

DR. CORNATZER: Was the choline given orally?

DR. WILGRAM: Yes, it was.

DR. COLIN C. LUCAS (*Toronto, Canada*): It has been proved that there is destruction of choline. However, I think that the amount of destruction either varies in different laboratories or is, perhaps, related to the basal diets that are used. In our work, we have obtained dramatic lipotropic effects from minute amounts of choline chloride, 0.02, 0.03, and 0.04 per cent.

Too much cannot be destroyed or there would be no effect. With 0.08 per cent, we obtained close to maximal effect. That is a minute amount of choline chloride, so maybe 2 to 5 or even 10 to 20 per cent is destroyed. I am not sure why Propert should find so much destroyed. Certainly, there is no evidence that our results are anything like that and, there is one paper (I do not recall the authors) that records minimal destruction.

DR. HARTROFT: Yes, there is another one besides that. I think that the importance of destruction has been overemphasized. However, it may occur in some laboratories, on some basal diets, or with some strange type of rats.

DR. ARTOM: The intestinal flora are probably involved.

DR. LUCAS: This is what I mean. With a certain basal type of diet intestinal flora may be active in destroying choline, but that is not inevitable.

DR. CHENOWETH: I question the absorption of choline. Has anyone ever determined blood levels of choline following single large oral doses to see whether it plateaus, whether absorption can be pushed past a certain blood level?

DR. ARTOM: When labeled choline is given, you can find quickly, after about an hour, that approximately 80 per cent is already converted into phospholipids. That happens when not too much choline is given. Of course, when larger doses of choline are given there is a decreasing rate of incorporation, but beginning with a reasonably low dose it is quite readily absorbed.

I think that the experiments of Stekol do arrive approximately at the same conclusion—that a large proportion of choline is quickly incorporated into phospholipids.

Lipotropic Factors in Transport of Cholesterol in Experimental Animals

COLIN C. LUCAS, PH.D.

AT Dr. Hartroft's suggestion I am reporting some of the experiments that Dr. Jessie Ridout and Dr. Jean Patterson and I did a few years ago as well as some more recent work.

We were not primarily interested in the blood picture but were working on fatty livers. Becoming aware of the suggestion that there was possibly a causal relationship between blood cholesterol levels and the incidence of atherosclerosis, we wondered just what choline, betaine or inositol might do in this situation.

Some of the rats from our usual liver studies were killed by exsanguination and the blood was analysed for cholesterol using the Schonheimer-Sperry-Webb[1] procedure. Thus, we obtained a single value at the end. Sometimes we pooled the samples. With no standard deviations, we did not know whether the differences were real or not. However, as time went on, certain facts and impressions emerged.

The time after the last meal at which one collects the blood sample is important, especially when the diet contains cholesterol and lipotropic agents. In our colony, when the blood of young rats (70 to 150 gm., maintained on a commercial ration) was taken eighteen hours or more after their last meal, total cholesterol in the serum was about 70 (±S.D. 11) mg. per 100 ml. with about 12 mg. in the free form; the rest was bound in ester form. Young males and females showed no significant difference.

When purified diets containing cholesterol and choline were fed to rats a distinct postprandial elevation in bound cholesterol was observed. The values were well above normal about two to four hours after eating and fell back in about sixteen to eighteen hours into the normal range and stayed there. No matter how much cholesterol was in the diet, even up to 1.6 per cent, if there was no choline in the diet, no significant elevation of the serum cholesterol occurred. When the food contained both choline and cholesterol there was a rather sharp rise which is related to the amount of cholesterol but not to the concentration of choline in the diet, as long as some was present.[2]

In the absence of choline no increase occurred in the serum cholesterol. This suggested, of course, that choline affects absorption of cholesterol but other explanations are possible and the data that we had available would not permit us to resolve this.

When the rats were kept on a hypolipotropic diet for any length of time the bound serum cholesterol fell progressively as the experiment was prolonged. In one experiment, after about six months the values had fallen to about 30 mg. per 100 ml. or even lower. However, addition of quite small amounts (0.02 per cent) of choline or betaine to the food maintained the serum cholesterol within the normal range. The administration of inositol, even at fairly high levels, failed completely to prevent this characteristic fall in the concentration of cholesterol in serum.

When choline was restored to the diet of rats with fatty livers and low serum cholesterol, the latter returned promptly from about 30 mg. per 100 ml. to 65 to 75, i.e., to the normal level. This happened within twenty-four hours, long before we could detect any chemical or histological change in the liver. Betaine had a similar

From the University of Toronto, Banting and Best Department of Medical Research, Toronto, Ontario, Canada.

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effect. The addition of inositol failed to bring about any rise.

Vitamin B_{12} was not tested under these conditions but both betaine and vitamin B_{12}, like choline, prevent the fall even when the animals are maintained on this ration for long periods. There were far too many uncontrolled variables in those early experiments. We tried to eliminate them one after another.

We had just started to investigate the effect of repeated withdrawal of blood upon serum cholesterol when I discovered that in several other laboratories, a gradual progressive change upward had been noted. We decided not to use the same animals for repeated testing because the results so obtained might be misleading unless we could find some better micro method. Fortunately, a more suitable method is now available, thanks to Zlatkis, Zak and their colleagues.[3,4] Using a reagent containing ferric chloride in concentrated sulfuric acid, reasonably accurate values can be obtained for total cholesterol on as little as 0.1 ml. of serum. We collect about 0.5 ml. of blood by snipping a tiny portion off the tail of a rat. If bleedings are limited to five or six in a period of forty-eight hours and the rat is not bled for a period of a couple of weeks, we find no progressive rise in the serum cholesterol.

When this method was used on the blood from large male rats (weighing between 400 to 500 gm.) the mean value for eighty-one samples from fifteen animals was 83 (± S.D. 19) mg. per 100 ml.

When these large rats, which had been on a standard chow diet, were given an injection of 16 mg. choline chloride, no change whatever occurred in the serum cholesterol at one, two, four, eight, twenty-four or forty-eight hours.

In one experiment with young rats (weighing 150 gm.) we studied the effect of ten different dietary fats fed for three weeks at the 20 per cent level in diets with six different amounts of choline upon the serum cholesterol.[5] The fats used were sunflower seed oil, corn oil, peanut oil, olive oil, Primex,® margarine, lard, beef fat, butter fat and coconut oil. In every case the serum cholesterol levels fell below normal when choline was omitted from the ration.

As little as 0.03 per cent choline in the food maintained the serum cholesterol values at about normal, with one or two exceptions. Rats getting butter and coconut oil required slightly over 0.03 per cent.

Higher doses of choline (up to 0.36 per cent) brought the serum cholesterol level back into the normal range and left it there. The different fats did not produce different levels of serum cholesterol, whether choline was present or not.

The big rats (those weighing 400 gm.) were maintained for a relatively long period (twenty-four weeks) on a hypolipotropic diet. The serum cholesterol level was then determined on the animals after they had been without food for eighteen hours. The values were low (30 to 60 mg. per 100 ml.), as we expected. The serums of the same rats were analyzed again after a few days, and a few weeks later, with remarkably good agreement, i.e., the values were on a plateau.

When these rats ate 1 to 5 gm. of choline-deficient food, there was no change whatever in the serum cholesterol level. With larger intakes (10 to 18 gm.) of choline-deficient food, a slight increase was occasionally observed. We would like to be able to say that regardless of the amount of choline-deficient food given, there was no increase in the serum cholesterol level. However, that may not be strictly true.

When similar rats with fatty livers and low serum cholesterol levels were given the identical food (a purified diet with about 16 per cent protein to which choline chloride had been added), the concentration of total cholesterol always rose promptly to within the normal range. Sometimes it took nearly twenty-four hours before the maximal value was reached but often it occurred within one to three hours. After remaining in the normal range for some hours, the values fell back slowly over several days. These "normalizing" effects of dietary choline were observed in rats fed a diet containing no cholesterol, hence the effect cannot in this case be due to alteration in the *absorption* of dietary cholesterol. These data suggest that one way in which choline can influence serum cholesterol levels is by affecting liver function, probably through *mobilization* of the cholesteryl esters piled up in the liver.

We have determined the changes, with time, of the concentration of total cholesterol in the serum of normal rats (males, weighing 400 gm.) following a single feeding of purified diets containing 1 per cent cholesterol, one diet containing 0.8 per cent choline chloride and one without choline. The animals ate about 25 gm. of food, which would provide about 250 mg. of cholesterol and 200 mg. choline chloride. With choline present, increases of 40 to 80 mg. per 100 ml. were observed, the maximal values occurring between one and five hours after removal of food; the values were usually back to normal within twenty-four hours. In the absence of choline, the increases in serum cholesterol were small (5 to 15 mg. per 100 ml.). The effects observed here could be due to either absorption or mobilization. The effect on mobilization seems to be limited, however, because dietary choline leads to values significantly above normal only when the diet contains cholesterol.

REFERENCES

1. Sperry, W. M. and Webb, M. A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. Biol. Chem.*, 187: 97, 1950.
2. Ridout, J. H., Patterson, J. M., Lucas, C. C. and Best, C. H. Effects of lipotropic substances on the cholesterol content of the serum of rats. *Biochem. J.*, 58: 306, 1954.
3. Zlatkis, A., Zak, B. and Boyle, A. J. A new method for the direct determination of serum cholesterol. *J. Lab. & Clin. Med.*, 41: 486, 1953.
4. Zak, B., Dickenman, R. C., White, E. G., Burnett, H. and Cherney, P. J. Rapid estimation of free and total cholesterol. *Am. J. Clin. Path.*, 24: 1307, 1954.
5. Best, C. H., Lucas, C. C., Patterson, J. M. and Ridout, J. H. Effects of different dietary fats and of choline on hepatic and serum lipids of rats. *Canad. J. Biochem. & Physiol.*, 36: 613, 1958.

DISCUSSION

Dr. Robert E. Olson (*Pittsburgh, Pennsylvania*): How about more prolonged fasting? Does it elevate the cholesterol level?

Dr. Lucas: The only time that we see the values going above normal is when there is choline and cholesterol in the diet or betaine and cholesterol, but even in these cases it comes back rather promptly.

It occurred to us that if choline did cause a rise above normal in certain situations that possibly one reason that would account for the alleged difference between animal fats and vegetable fats (one being supposedly more lethal than the other in causing atherosclerosis) was that it might have something to do with the height of this peak. We made a comparison between lard and corn oil under these circumstances. The ration contained 25 per cent of the fat (lard or corn oil), 2 per cent of cholesterol, 0.3 per cent of choline chloride, and the protein level was 16 per cent.

These diets were fed to groups of fifteen rats for three weeks and then samples of blood were taken terminally. The first experiment was carried out using the Schonheimer-Sperry-Webb procedure. We took samples from different groups of rats at four, eight and twenty-four hours after the removal of food. We were not really comparing the same rats, but what we hoped were comparable rats.

We could see no difference whatever between the two groups. We were not entirely happy with the results, however, since we were dealing with different rats. More recently, after becoming familiar with the Zak method, we took sequential samples of blood from the tail vein of the same rat at one, two, four, eight, twelve, twenty-four and forty-eight hours. At no time was there a significant difference between the serum cholesterol levels of the rats fed corn oil from those fed lard. This group had been kept on the diet for three weeks.

Dr. George F. Wilgram (*Toronto, Canada*): It may be important to mention that the rats, prior to the taking of the blood, were starved for twenty-four hours. They were hungry and when food was given they all gobbled it up and were more or less in the same state of fat absorption.

Phosphatides in Experimental Atheroma

MEYER FRIEDMAN, M.D.*

PLASMA phospholipid has been found elevated in almost all types of endogenous hypercholesteremia and it has always been thought that the phospholipid rise was concomitant with that of cholesterol or possibly compensatory for the rise of cholesterol. Etiologically speaking, phospholipid is possibly discharged into the blood to keep the excess cholesterol soluble in the serum.

It is easier to carry out a cholesterol analysis than a phospholipid analysis, so perhaps cholesterol elevations took preference in our thinking. However, we decided to study whether it was possible to determine if a hypercholesteremic state was due to a primary rise of plasma phospholipid or triglyceride, or both.

We thought this might be possible first, because phospholipid is a more mobile substance as far as blood plasma is concerned, and second, any attempt to find a change in the rate of synthesis of cholesterol in hypercholesteremic states in both the human being and the experimental animal has resulted in the findings that there is no increase. There is, on the contrary, a slight decrease or no change.

If a primary phospholipid rise caused hypercholesteremia it should be possible to cause the plasma phospholipid to rise in a normal animal and thus obtain a hypercholesteremic state. We found that when the normal rat was infused with phospholipid (2 per cent solution) at the rate of 0.5 cc. per hour, within a few hours the plasma cholesterol rose, and as the plasma phospholipid became higher the cholesterol followed suit.

In our studies, the infusion of phospholipid had no effect on either the plasma cholic acid or the plasma triglyceride. It elevated only the plasma cholesterol, and this elevation was only apparent after two or three hours.

The next problem was to determine whether or not a primary rise in plasma triglyceride would also elevate plasma cholesterol. There are some hypercholesteremic states, such as occur in nephrosis and after the injection of triton (trinitrotoluene) and in diabetic acidosis, in which hyperlipemia and a hypercholesteremia result.

We subjected the normal rat to an infusion of fat at a high level. Within a short time both the plasma cholesterol and the phospholipid rose. We found an initial induced rise of triglyceride will cause a rise of both of these other lipids. But if one elevates the plasma phospholipid first, a rise in cholesterol, but not in plasma triglyceride, is observed.

We believe that all forms of endogenous hypercholesteremia are secondary phenomena. There is nothing initially wrong with the cholesterol metabolism *per se*, at least in the beginning, but it represents the response of the body to a prior rise of either phospholipid or triglyceride, or both.

Another consideration is biliary obstruction. An accumulation of bile acid in plasma is the first manifestation of biliary obstruction. Within fifteen minutes after the bile duct of a rat is ligated the plasma cholic acid rises and it precedes by hours any rise of cholesterol, and by minutes any accumulation of excess phospholipid. In a short time (by a presently unknown mechanism) the bile acid effects an accumulation of phospholipid.

In other words, if a normal animal is infused with bile acid even without biliary obstruction and the bile acid is elevated in plasma by giving bile acid so rapidly that the liver cannot excrete sufficient bile acid to prevent its rise in the

From the Harold Brunn Institute, Mount Zion Hospital, San Francisco, California.

* Director, Harold Brunn Institute.

Presented at the First Symposium of the Lipotropic Research Foundation, April 13, 1958, Philadelphia, Pennsylvania.

plasma, the phospholipid rises, followed within a short time by a rise in plasma cholesterol.

The elevation in plasma cholesterol apparently stems from other tissue sources besides the liver. We were misled by our own previous work to believe that the liver was the only source of plasma cholesterol in hypercholesteremia, and other investigators also came to that conclusion.

We do not believe now that only the liver is capable of supplying large amounts of cholesterol to the plasma. Removal of the liver removes the source of the blood's phospholipid and since a cholesterol rise depends on a preceding rise in phospholipid, removal of the liver does lead to a failure of cholesterol to increase in the blood after supposedly hypercholesteremic procedures. This is not due to the necessity of cholesterol being furnished to the blood only by the liver. It is probably occasioned by the failure of the animal without a liver to supply phospholipid to its blood.

After injection of triton, a failure of the clearing mechanism is seen. We believe, as did the original workers, that this happens because administration of triton interferes with the acceptance by plasma albumin of the fatty acids resulting from hydrolysis. Whether this is due to the sequestration of the triglycerides by triton or the physiologic sequestration of albumin by triton is not known at the present time. However, we do know that whenever there is an accumulation of triglycerides occurring after administration of triton (which occurs within the first few hours), it is this triglyceride rise which then mobilizes the tissue cholesterol everywhere in the body to effect a hypercholesteremia, whether or not the liver has been removed.

Various workers thought that the presence at the liver was necessary for the occurrence of hypercholesteremia after injection of triton. However, if one injects one dose of triglyceride and also gives triton to a hepatectomized animal, the animal becomes and remains hyperlipemic and, as a consequence, becomes hypercholesteremic in six hours. On the other hand, if one gives triglyceride without triton to an animal whose liver has been removed, it rapidly rids its serum of the fat, but in the presence of triton it cannot dispose of the triglyceride. As a result the animal becomes hypercholesteremic within six to eight hours.

It is our opinion that in nephrosis, when the albumin falls below 1 gm. per 100 ml., there is no receptor for the fatty acids coming from the hydrolysis of fats. Therefore, the triglyceride remains in the plasma and when it accumulates it becomes a cholesterol receptor and retainer.

We also believe that a certain amount of phospholipid or triglyceride, or both, can hold only a certain amount of cholesterol in plasma, and if a nephrotic animal whose triglyceride component is saturated with cholesterol is given another injection of cholesterol, it will dispose of that excess cholesterol as easily as a normal rat. The cholesterol goes directly to the liver of the animal for removal because, seemingly, the animal has no way of retaining the cholesterol when its triglyceride component is saturated. The mechanism is unknown.

This occurs also after injection of triton. Thus, if an animal is given triton followed immediately by administration of a soluble form of cholesterol, the animal will not receive any cholesterol from its tissues but instead will retain the administered excess cholesterol in its plasma. In twenty-four hours, the same rise in blood cholesterol is observed in an animal given an initial injection of cholesterol plus triton as in an animal given triton alone.

Studies in our laboratory show that almost all lecithins were capable of inducing a cholesteremic effect, except lecithin from the duck's egg. Cephalin as well as inositol and synthetic lecithins provided a hypercholesteremic effect. The animal whose liver has been removed also becomes hypercholesteremic after infusion of phospholipids.

We have found that there are two tissues with a reduced cholesterol content after phosphatide infusion: (1) the liver of an animal that is fed cholesterol in excess; and (2) the adrenal gland of a starved animal.

If cholesterol could be removed from various depots by phosphatide infusion, the possibility existed that we might be able to remove cholesterol from an atherosclerotic plaque. Therefore, using fourteen rabbits, seven were infused

twice a week (eight to eleven infusions before autopsy) with a crude extract of brain lecithin, and seven others served as control subjects. The extract (a very potent cholesterol mobilizer), containing various types of lecithins and cephalins was not toxic in this particular group of animals although it did contain a high content of lysolecithin. During each infusion the plasma phospholipid would be artificially elevated and the plasma cholesterol would usually rise from 50 to 250 mg. per 100 ml. in six hours in the rabbit. (If phosphatides are administered too frequently, e.g., three times a week, a chronic hypercholesteremia results which will continue as long as infusions of phosphatides are given.)

Results of phospholipid infusions in our group of fourteen animals after three months were as follows: The degree of aortic atherosclerosis was 4.9 in the control rabbits, and only 2.7 in the rabbits infused with phosphatide. The actual cholesterol and fat analyses of the aortas indicated a 51 per cent difference in the aortic cholesterol and a 37 per cent difference in the total lipid.

These apparently therapeutic results obtained with infusion of a crude phosphatide suggested that perhaps a means was at hand that might reverse the atherosclerotic process. However, we were handicapped by the tediousness of preparing the atherosclerotic animal. Cholesterol must be fed for two to three months and then cholesterol feeding must be stopped for another two months for the animal to become normocholesteremic. Therefore, we adopted the Higginbotham technic. This consists of removing an atherosclerotic aorta from a hypercholesteremic animal and cutting it into small segements. Every other segment is given to a control animal and the animal to be infused is given the alternate segment. Such segments are placed in the interior chamber of the eye. One can easily perform fifteen such corneal implants in one day.

Our experiences do not agree with Dr. Higginbotham's statement that the intima of even an atherosclerotic plaque in the cornea will be vascularized. We do not believe there is any vascularization of the intima when we place the adventitial side of the aorta directly upon the iris, which is richly supplied with blood.

One week after implantation, we infused half of the animals with a phosphatide suspension (5 per cent) for approximately six to eight hours, twice a week for four to six weeks. The corneal implants then were removed, a section taken of each and stained with Sudan III. The remainder of each corneal implant was then analyzed for cholesterol. Similar procedures were carried out on the control rabbits, on which corneal implant had not been performed.

The rabbits withstood the infusions very well and little or no toxicity was observed. During infusions, the plasma phospholipid is elevated six to ten times above the normal value, effecting a four- to sixfold rise in the cholesterol level. Between infusions, both lipid levels returned to normal.

This work has just begun and it is not certain whether or not phosphatide infusions hasten the disappearance of cholesterol from such implants. Unlike aortic atherosclerosis, these implants naturally lose their cholesterol very quickly in a period of four to eight weeks. It remains to be seen whether or not phosphatide therapy will alter this natural rate of disappearance.

DISCUSSION

DR. W. STANLEY HARTROFT (*St. Louis, Missouri*): These are exciting results that Dr. Friedman has just given us. It is interesting that treated plaques with the fat removed contain only calcium and the calcium is all in the media. That is the picture of Mönckeberg's sclerosis. Have the plaques progressed from atheroma to Mönckeberg's sclerosis? If so, it has tremendous implications.

DR. FRIEDMAN: There is sometimes calcification of the intima but it is mostly in the media.

DR. HARTROFT: You demonstrated mainly medial calcifications; therefore you have transformed an atheromatous plaque into Mönckeberg's sclerosis. If this is accepted on its face value it will greatly affect our thinking in pathologic study.

Role of Vitamins in Lipid Metabolism

BACON F. CHOW, PH.D.*

THE data presented herein have already been reported in part by Dr. Ling.[1] However, some unpublished work by Dr. Hsu[2] and myself is also included.

Ling[3] previously showed that the lipid content of a vitamin B_{12}-deficient rat is 4.4 per cent, and that of the vitamin B_{12}-treated rat 16.8 per cent. The difference in the fat content of these two groups of animals may be attributed to vitamin B_{12} deficiencies or to a more general phenomenon, such as inanition or poor appetite. For example, it should be pointed out that the mean body weight of the deficient animals was approximately 84 gm. and that of the treated animals, 200 gm. The food consumption of the two groups was different.

In subsequent experiments, two groups of rats were pair-fed. One can pair-feed these animals in two ways, that is, limit the food intake of the vitamin B_{12} treated group to the amount of food taken by the vitamin B_{12}-deficient one; or, feed *ad libitum* the amount of food and calories taken by the vitamin B_{12}-treated group and force-feed the vitamin B_{12}-deficient group to the same extent.

When these animals were pair-fed according to the amount taken in by the vitamin B_{12}-deficient group, then we found that vitamin B_{12} had no effect, and the weight gain of the two groups was essentially the same.

On the other hand, when we pair-fed according to the amount consumed by the vitamin B_{12}-treated animals—and to achieve this goal we had to use a liquid diet consisting of oil, emulsified fat, sucrose and soybean protein—we found that there was a marked difference again in the lipid composition of the carcass, and most of the fat in the food consumed by the vitamin B_{12}-deficient group was excreted in the feces.

As a further control on the effect of body weight, a third group of rats was used. It consisted of vitamin B_{12}-treated rats that had been starved so that their weight had been considerably reduced. Upon feeding with sufficient calories they gained much weight and the fat content was the same as that of the vitamin B_{12}-treated group. This type of control study is essential if we are to be reasonably certain that the effect is due to vitamin deficiency.

Ling[1] demonstrated that vitamin B_{12}-deficient animals show a small amount of carcass lipid and phospholipids. Hsu[2] recently found that the serum cholesterol content in plasma and adrenals of vitamin B_{12}-deficient animals was elevated almost twofold. When these animals were treated with vitamin B_{12}, the serum cholesterol level was brought down to almost normal. Addition of an excessive amount of pyridoxine had no effect.

Of interest is that the administration of vitamin B_{12}, 10 μg. per day orally or 10 μg. once weekly subcutaneously, results in a definite decrease in plasma cholesterol.

The most effective way of correcting hypercholesteremia in rats caused by a deficiency of vitamin B_{12} is by the subcutaneous administration of vitamin B_{12}, 10 μg. per day, plus the oral administration of 10 μg. of vitamin B_{12} with 100 mg. of sorbitol. Sorbitol[4] has been shown to be an agent which will enhance absorption of vitamin B_{12} under some experimental conditions.

When vitamin B_{12} is given orally in small doses, only a certain portion of it is absorbed. The absorbed portion of this vitamin, unlike

From The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland.

* Associate Professor of Biochemistry.

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other ones, is not distributed evenly to all organs. The vitamin can be transported[5] to certain target organs such as liver, adrenals and pancreas, but it is retained in these depots so tenaciously that it is not available for redistribution to other organs. When vitamin B_{12} is given as an injection even in large quantities, most of it is excreted rapidly and certain target organs do not have a chance to retain it.

The addition of sorbitol has been shown to enhance the absorption of vitamin B_{12}, not only does it raise the serum level, but vitamin B_{12} can also go into certain organs (eyes and brain) which cannot be reached even by the injection of large doses of vitamin B_{12}.

We were interested in determining whether the administration of vitamin B_{12} would have any effect on lowering the high serum cholesterol levels of old people. In a study conducted by other workers, it was found that a certain mixture of lipotropic agents (Liptril[6]) possessed the property of lowering serum cholesterol levels. In view of our findings, we thought it might be possible to decrease the cholesterol levels in these older subjects by injections of vitamin B_{12}. Our first attempt to do so was unsuccessful.

In view of these results, we considered the possibility of lowering the serum cholesterol levels by administering vitamin B_{12} with sorbitol orally. It was found[7] that the administration of a mixture of sorbitol and vitamin B_{12} resulted in an increase in vitamin B_{12} serum level and a concomitant decrease in the cholesterol level.

REFERENCES

1. Ling, C. T. and Chow, B. F. Effect of vitamin B_{12} on the body composition of rats. *J. Biol. Chem.*, 198: 439, 1952.
2. Hsu, J. M. and Chow, B. F. Vitamin B_{12} deficiency and hypercholesteremia. *Fed. Proc.*, 16: 63, 1957.
3. Ling, C. T. and Chow, B. F. The influence of vitamin B_{12} on carbohydrate and lipid metabolism. *J. Biol. Chem.*, 206: 797, 1954.
4. Chow, B. F., Meier, P. and Free, S. M., Jr. Absorption of vitamin B_{12} enhanced by D-sorbitol. *Am. J. Clin. Nutrition*, 6: 30, 1958.
5. Lang, C. A., Harte, R. A., Conley, C. L. and Chow, B. F. Retention of crystalline vitamin B_{12} by healthy male individuals following intramuscular injection. *J. Nutrition*, 46: 215, 1952.
6. Blumberg, D., Fischbach, M. W. and Zisserman, L. Preliminary evaluation of a non-dietary regimen for reducing cholesterol levels in the aged. *J. Am. Geriatrics Soc.*, 4: 276, 1956.
7. Thomas, C. B. and Eisenberg, F. F. Variability of cholesterol levels in individual Johns Hopkins medical students, with observations on stopping smoking, vitamin B_{12} administration and acute infection. *Bull. Johns Hopkins Hosp.*, 105: 14, 1959.

DISCUSSION

Dr. George F. Wilgram (*Toronto, Canada*): Dr. Friedman, you said you believed that the hypercholesteremia under the conditions of your experiment is caused by, may we use the word, "secretion" of cholesterol into the blood from sources other than the liver. Is this correct?

Dr. Meyer Friedman (*San Francisco, California*): Yes.

Dr. Wilgram: Could you elucidate on what you believe to be the role of the liver in the contribution of cholesterol to the blood under these conditions?

Dr. Friedman: This is about what we believe at the present stage of our work: if the animal is pre-fed a high cholesterol diet, a decline in liver cholesterol occurs after infusion of phosphatide. This is accompanied by marked hypercholesteremia. At the same time, there is also a marked decline in adrenal cholesterol. When the animals receive no cholesterol in the diet, the cholesterol content of the liver is increased and that of the adrenal decreased during the period of the phosphatide-induced hypercholesteremia. We also found a similar hypercholesteremia after phosphatide infusion in the liverless animal which leads us to believe that it is not the normal function of the liver to supply the blood with cholesterol.

Dr. Sachs will also discuss this question, since he has too been working with a phosphatide and getting a rise of cholesterol in two or three minutes.

I do not know exactly where the cholesterol is coming from; however when we feed cholesterol it is obtainable from the liver and the adrenal.

Dr. Wilgram: How then does this tie in with the clinically well known statement of Thannhauser about thirty years ago that in severe liver disease the first real sign that can be detected in the blood is a decrease of the cholesterol esters? I think most people would agree that there is some correlation between the disease and the drop in the cholesterol esters, presuming that the cholesterol has been esterified and released from the liver.

Dr. Friedman: I think we went to long lengths to show that the liver was the chief site of cholesterol esterification but that does not mean that quantitatively it is supplying an excess cholesterol for the blood. I think there is an easy transfer of cholesterol back and forth.

I believe the liver cell has a certain amount of cholesterol which it is not going to give up to the plasma, irrespective of what you do. I think it will give up phosphatides and triglycerides.

I am not prepared now to discuss this because it really does not make any difference to me whether or not the liver produces a lot of cholesterol. As a matter of fact, half of those in my lab do not believe it does.

DR. WILGRAM: I think Dr. Lucas' work and also Dr. Olson's corroborate the fact that in the choline-deficient animal, when choline is fed, the cholesterol increases in the blood. But where does this come from, if not the liver? It decreases in the liver at the same time it increases in the blood.

DR. FRIEDMAN: Dr. Worthason has shown that the liver is the only organ in the body that has a net increase in cholesterol. Never has anyone found an excess, showing that the liver could manufacture cholesterol in excess. It is just short of turnover.

Now I do not know, and I do not like to discredit my own early work either, because we thought we were pretty good in showing that the liver was the chief source of cholesterol in the blood. However I do not care. I would like you to show me that this statement is wrong.

DR. W. STANLEY HARTROFT (*St. Louis, Missouri*): I think this view of yours is very stimulating because it makes us look in other directions.

Dr. Lucas, would you tell us more about this aspect? Would you also tell us where the cholesterol goes in a choline-deficient animal when the serum cholesterol falls; and where it comes from when choline is restored to the diet and the serum cholesterol rises; and where it is delivered?

DR. C. C. LUCAS (*Toronto, Canada*): I wish I knew the answers. The actual fall, even the blood level was about 200 and fell back to 80, is not many milligrams compared to the amount of cholesterol in the rest of the body. You would have to be a pretty tricky analyst unless you were going to use tracer technics in order to pick that up. We have not applied tracer technics so I could not tell you where it goes.

DR. HARTROFT: If it goes into the aorta we would be very interested.

DR. W. E. CORNATZER (*Grand Forks, North Dakota*): In the effect of the rise in cholesterol caused by giving choline to the choline-deficient animal, I would like to point out that in the choline-deficient animal given either a dose of choline, a single dose of oil or a single dose of oil plus choline, the greatest stimulation of phospholipid synthesis occurs in the intestinal mucosa and in the liver.

(This is our initial work with Dr. Artom.) We obtain not only a rise in serum cholesterol following a given dose of choline or choline plus food containing some oil, but also a rise in phospholipids above that which is obtained when giving oil alone, choline alone, or oil plus choline.

Now if this occurs in the liver, I am sure it occurs in the plasma, although we did not study the plasma at that time because from all other work the plasma simply reflects that which is taking place in the liver.

DR. HARTROFT: What do you think the increase in phospholipid synthesis in the intestines means and why shoud it occur there?

DR. CORNATZER: Probably increased metabolism, because we do not think it is involved in absorption.

DR. C. ARTOM (*Winston-Salem, North Carolina*): We are not sure.

DR. CORNATZER: That is so. The pendulum has swung away from such an explanation and may be back again if you interpret Reiser's experiments, so it depends on what the exact mechanism is. Dr. Artom, I am sure, can answer this question.

DR. ARTOM: It is an old question. It was discussed twenty or twenty-five years ago with Dr. Verta, who was at that time very enthusiastic about the participation of phospholipids in the absorption of fat. I think if we try to calculate the increase in the synthesis of these phospholipids, certainly the increase will not justify that all the fatty acids pass through the stage of phospholipids during absorption. That is part of it.

DR. LUCAS: There appears to be quite a large discrepancy in the calculation.

DR. ARTOM: Yes, it is too literal. In other words, an increase in the rate of synthesis would not be high enough to justify the absorption of fats which occurs normally, so certainly they are not all absorbed as phospholipids.

It is certainly only a secondary pathway but the secondary pathway may become important when you have some impairment in the major pathways. That might be part of the process in the absorption but I think we have to leave the question as it is, in other words, still somewhat unsolved.

DR. LUCAS: I think it is possible that the observed phospholipid turnover may not be an obligatory part of the system, but a parallel process, something that is going on simultaneously that is important.

DR. ARTOM: It could perhaps be just the expression of an increased metabolism.

DR. HARTROFT: Would some one comment on the phenomenon that enables ethanolamine to increase phospholipid turnover in the liver just as choline does, without being lipotropic.

DR. ARTOM: I do not know exactly, but I would say that there is one point perhaps that can reconcile these two differences. In the liver we have lecithins, and cephalin, including ethanolamine; in the plasma we have only the lecithins or very little phospholipid ethanolamine. It might well be that when the one is reflected in the phospholipids and the other is not, it is because it remains in the liver.

What the real mechanism of this increased synthesis is, I do not know. Perhaps it is just a mass effect of overflowing phospholipid from the liver, with the compound that we give. We have so many compounds that increase phospholipid synthesis and none of them

certainly is directly or easily explained as a lipotropic substance.

DR. LUCAS: In Dr. Cornatzer's discussion of the lipotropic effect of methionine, he noted that at the 6 per cent level a marked change in the level of lipids occurred. I do not think I would call that a lipotropic effect of methionine. In some of our work (using quite a few different basal diets), it we added much over 1 per cent of methionine to the diet we began to get a cutback on food consumption and a decrease in weight gain, until finally we got a weight loss, just as Dr. Cornatzer showed. I do not think you can get a fatty liver unless enough calories are given to supply the body needs, and a little excess.

I believe 5 gm. a day loss of weight does not show a lipotropic effect. I would call it a toxic effect.

DR. CORNATZER: We interpret this as an antilipotropic effect, showing the loss in weight and decreased food consumption.

DR. LUCAS: You could not call it an antilipotropic effect in one sense because the liver fat is falling, but it is falling because of diminished fat intake.

DR. CORNATZER: That is right.

DR. HARTROFT: You could not describe that as an unique action of methionine?

DR. CORNATZER: No.

DR. HARTROFT: Because if you can do anything to the animal that will cause a decrease in food intake, the amount of fat will decrease.

DR. WILGRAM: Dr. Friedman, I was very much impressed by your closing remarks when you described the implantation of a segment of the aorta into the anterior chamber of the eye by the Higginbotham technic and observed *in vivo*, so to speak, the atherogenic process. Normally, does the aorta graft on the iris or not? That is, does the adventitia adhere to the iris?

DR. FRIEDMAN: Yes. It sticks and it does not die; that is, it does not form a necrotic mass for weeks and months, up to even six months. I have not done it for six months but Dr. Higginbotham has and rarely does he find a normal aortic plaque calcified like the one I showed you today.

DR. WILGRAM: And you believe that the cholesterol deposition under those circumstances occurs through the capillaries?

DR. FRIEDMAN: Of the iris, yes. And from the adventitious side, not the intimal side.

DR. WILGRAM: You do not believe that any cholesterol transgresses the blood barrier and goes into the fluid? That would be unlikely, would it not?

DR. FRIEDMAN: It surely would. We have never noticed any opacity or turbidity of that fluid and we do not notice the intimal side ever acquiring a deposit unless the iris tissue comes around and covers the intimal side.

DR. HARTROFT: I think this technic is certainly one that more people will find interesting.

The Effect of Safflower Oil on the Nature of Serum Cholesterol Esters

R. B. ALFIN-SLATER, PH.D.* AND PAUL JORDAN, M.D.†

MANY investigators have reported that when vegetable oils rich in essential fatty acids are administered to subjects with hypercholesterolemia, they cause a decrease in serum cholesterol levels. Usually, the vegetable fat is given as the sole source of fat in a diet with carefully controlled concentrations of other nutrients. The purpose of this investigation was to determine whether safflower oil, a vegetable oil containing high concentrations of essential fatty acids, would still be effective in reducing serum cholesterol levels if given to hypercholesterolemic patients as a supplement to their usual diets and, if so, to find out how soon this effect could be achieved, whether relatively small doses of the oil could be used with any success, and whether a decrease in serum cholesterol concentration was accompanied by an increase in unsaturation of the fatty acids contained in the cholesterol esters.

MATERIAL AND METHODS

Eight ambulatory, male patients with hypercholesterolemia, from the Veterans Administration Center, Los Angeles, were selected. Their serum cholesterol levels were measured during a control period at which time they were fed usual hospital rations. At the termination of the control period, a safflower oil supplement was administered orally three times daily after meals. The amount of the dose and the experimental period was varied. In the first series of experiments 7.5 ml. per dose (a total

TABLE I
Relationship Between Plasma Cholesterol Levels and Iodine Value of Cholesterol Ester Fatty Acids

Control Diet		Safflower Oil Diet	
Cholesterol (mg. %)	Iodine Value	Cholesterol (mg. %)	Iodine Value
Patient H. S.			
354	...	272	188
373	104	245	186
323	124	...	...
Patient R. B.			
290	...	291	184
286	108	288	124
289	112	...	...
Patient H. B.			
345	...	259	184
355	...	310	146
318	114	...	...
Patient M. C.			
267	143	251	158
264	124	267	184
271	160	...	...
Patient F. W.			
258	100	253	170
254	140	256	161
270	156	...	...
Patient D. R.			
273	123	217	156
260	113	...	...
Patient E. C.			
488	97	414	155
425	116	...	...
Patient A. A.			
297	129	272	119
260	113	...	...

From the Department of Biochemistry and Nutrition, University of Southern California School of Medicine, and the Surgical Service, Veterans Administration Center, Los Angeles, California.

* Department of Home Economics, University of Southern California. † Department of Surgery, University of Florida, Gainesville, Florida.

of 22.5 ml. or approximately 21 gm.) was given during the first period of supplementation. Thereafter, the dose was decreased to 5 ml. three times per day after meals. Each experimental period lasting from two to four weeks was preceded by and then followed by a control period of two to four weeks during which supplementation was discontinued. Blood was drawn from the patients at the termination of each experimental and control period.

Plasma cholesterol determinations were made and the cholesterol esters were separated from the other lipid components of the blood by silicic acid chromatography. The cholesterol esters were eluted, saponified to obtain the fatty acids, and iodine values were determined on the mixed fatty acids.

RESULTS

The results are shown in Table I.

SUMMARY

A decrease in serum cholesterol levels was achieved in half the patients receiving the safflower oil supplements. In these patients, a return to unsupplemented hospital rations was accompanied by an increase in the serum cholesterol level. Attempts to correlate this decrease in serum cholesterol level with an increase in unsaturation of the cholesterol ester fatty acids were unsuccessful in that the iodine value of the fatty acids was increased after safflower oil supplementation even when the serum cholesterol levels were unaffected (Table I). It is possible that a longer experimental period with larger supplements of safflower oil might have showed more positive results. However, a response of 50 per cent is better than would be expected if this were entirely due to chance. It can be concluded that relatively small amounts of safflower oil, when supplemented to an ordinary diet, are effective in decreasing serum cholesterol levels in some persons in relatively short periods of time. Whether a positive response to this therapy is dependent on the essential fatty acid stores and/or requirement of a patient has not been shown in this investigation.

Radioactive Fat Absorption Patterns in Obesity and Coronary Disease

DONALD BERKOWITZ, M.D.*

MANY attempts have been made to attribute the genesis of atherosclerosis in some manner to an error in fat metabolism. However, the results to date have been inconclusive and frequently conflicting. With the availability of newer technics utilizing radioactive substances it may be anticipated that more informative data will be forthcoming to help elucidate this basic problem.

During the past year we have been using radioactive triolein to study the absorption and utilization of fat in normal subjects and in patients with various diseases.[1–4] This report is concerned with the results obtained in patients with coronary artery disease and/or hypercholesteremia. In addition, because of the frequent association of coronary disease with obesity, the response of a group of obese subjects to a tagged fat meal has also been studied.

PROCEDURE

The procedure consists essentially of the administration of a test meal containing 25 μc. of I^{131} - triolein in a total volume of 1 ml. of peanut oil per kilogram of body weight. Samples of venous blood are taken at frequent intervals until a maximum radioactivity level has been reached, and then twenty-four hours later. A 2 ml. aliquot of unclotted blood is counted in a scintillation well counter (whole blood radioactivity). Another aliquot, treated with potassium iodide and trichloracetic acid to separate the lipoprotein-bound iodine, is also assayed for radioactivity. The total blood volume is assumed to be 7.2 per cent of the body weight. With the given activity in the 2 ml. portions and the calculated total blood volume, the total whole blood and lipid blood radioactivity can be determined and expressed as a percentage of the initially ingested fat.

The total urine output is collected for twenty-four hours after the meal, and also is assayed. In some cases, stools are collected over a seventy-two–hour period.

After the radioactive triolein is ingested, it enters the intestinal tract and is then absorbed. The radioactive iodine which circulates in the blood consists of two fractions. The first is contained in the fat which is in transport as lipoprotein. Normally, this portion does not exceed 50 per cent of the total radioactivity.

The second fraction is derived from the splitting of the iodine-fatty acid bond during utilization. The concentration of this inorganic fraction depends on the rate at which the fat is utilized and the speed of renal excretion. The influence of the thyroid on this fraction is negated by the use of blocking agents prior to the test meal.

The essential features of the blood radioactivity curves include (1) the time of maximum blood radioactivity; (2) the magnitude of this maximum value; (3) the twenty-four–hour activity level; and (4) the organic blood/whole blood radioactivity ratio. Although these values are only a partial reflection of the complete metabolic cycle which ensues after a

From the Department of Medicine, Hahnemann Medical College, and the Albert Einstein Medical Center, Philadelphia, Pennsylvania.

* Instructor in Medicine, Hahnemann Medical College and Attending Gastroenterologist, the Albert Einstein Medical Center.

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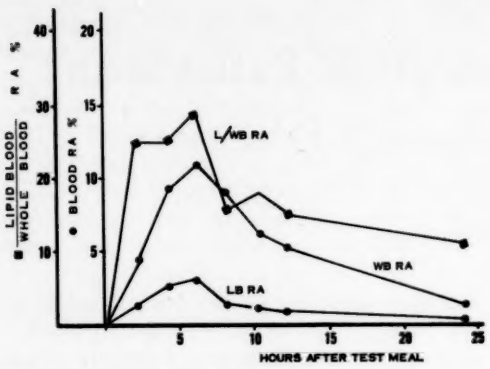

FIG. 1. Mean radioactive fat tolerance curves in a normal subject. In this and other illustrations, WB = whole blood, LB = lipid blood, RA = radioactivity. (From: LIKOFF, W., BERKOWITZ, D., WOLDOW, A., JACOBS, A. G. and SKLAROFF, D. M. *Circulation*, 18: 1118, 1958.[1])

labeled fat test meal, they appear to be the most informative.

RESULTS

In our normal control subject, after ingestion of a triolein test meal, the mean whole blood radioactivity usually increased to a peak value of 11.7 per cent (lipid blood value, 3.7 per cent) by the sixth hour and then gradually declined so that after twenty-four hours approximately 2 per cent (lipid blood value, 0.2 per cent) remained. The organic fraction represented less than 50 per cent of the total (Fig. 1). Twenty-four–hour urine collections contained between 20 and 40 per cent of the administered radioactive substance, and the average fecal radioactivity over this period is less than 2 per cent.

In our patients with coronary artery disease, the curves demonstrated marked differences from those in the normal subjects. Characteristic were unusually high blood levels of total and lipid blood radioactivity, delays in attaining these concentrations, and abnormal twenty-four–hour retention (Fig. 2). In addition, the lipid/whole blood activity ratio in many, showed that a fraction was present in the organic portion.

In a group of twenty-five obese female subjects (weighing 175 to 300 pounds) we again noted the elevated radioactivity values at the peak time and after twenty-four hours. This

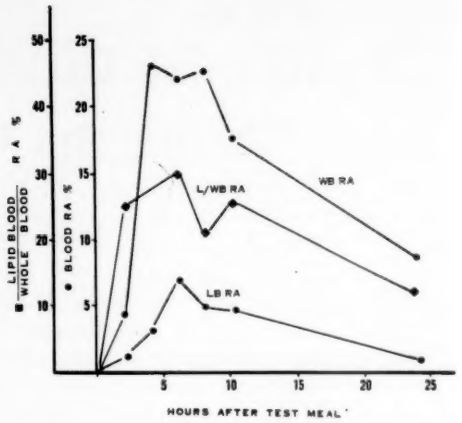

FIG. 2. Abnormal radioactive fat tolerance curves in patient with coronary artery disease.

was reflected in both the whole blood and the lipid blood fractions (Fig. 3).

COMMENTS

It would thus appear that patients with coronary artery disease, as well as obese subjects, demonstrate similar abnormalities after a radioactive fat tolerance test as compared to normal control subjects. Any attempt to explain these absorptive patterns presupposes a knowledge of various factors concerned therewith.

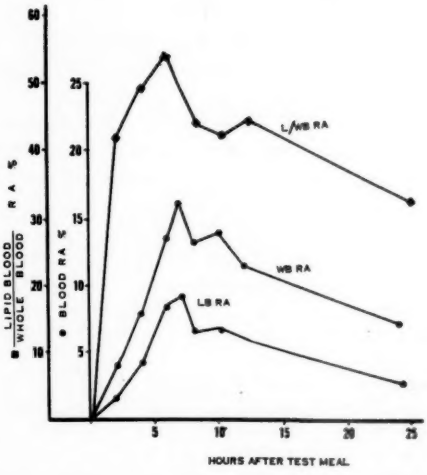

FIG. 3. Mean radioactive fat tolerance curves in obesity without coronary disease.

Under normal conditions, at least 97 per cent of the radioactive fat is absorbed within twenty-four hours. The amount present in the blood at any one precise moment, however, depends upon additional factors, notably the rate of utilization and the size of the fat pool. It is possible that an increased rate of absorption may be a factor in these higher blood curves. This could result from a decreased intestinal transit time allowing more time for the fat to be exposed to the absorbing surface, or else, a primary mucosal phenomenon may be responsible. Data showing that the intestinal mucosa is able to absorb abnormally high amounts of a basic foodstuff in certain conditions have already been advanced by Mayer.[5] He found that in certain mice with experimentally produced obesity, an increased rate of glucose absorption may be present—an apparent compensatory mechanism for the hyperphagia. On the other hand, abnormally high blood radioactivity levels may be the consequence of some derangement of fat transport. The delay in the disappearance of the isotope after twenty-four hours may have a similar explanation.

Thus far, the role of the pre-existing fat pool has not been taken into account. It may be that in those cases associated with hypercholesteremia, the abnormal curves are a result of the hyperlipemia and its attendant chemical dilution. Further studies correlating the basal triglyceride and total fat values are now in progress and may help to clarify this problem.[3]

The inability to define the precise mechanisms which are responsible for the abnormal radioactive fat tolerance curves we have found in these studies makes it difficult to offer any major conclusions. However, it would appear that the isotopic fat technic offers an excellent means for studying lipid metabolism. With this material it is possible to follow the fate of ingested fat in a much simpler and more precise manner than was heretofore available.

If further studies substantiate our preliminary data, it may be possible to utilize this procedure as a screening device to predict the presence of coronary disease in asymptomatic persons.

REFERENCES

1. Likoff, W., Berkowtz, D., Woldow, A., Jacobs, A. G. and Sklaroff, D. M. Radioactive fat absorption patterns—their significance in coronary artery atherosclerosis. *Circulation*, 18: 1118, 1958.
2. Berkowitz, D., Sklaroff, D. M., Woldow, A., Jacobs A. G. and Likoff, W. Blood absorptive patterns of isotopically-labelled fat and fatty acid. *Ann. Int. Med.*, 50: 247, 1959.
3. Berkowitz, D., Likoff, W. and Spitzer, J. J. Serum triglyceride, unesterified fatty acid, and radioactive fat tolerance studies. *Fed. Proc.*, 18: 191, 1959.
4. Berkowitz, D., Sklaroff, D. and Likoff, W. Radioactive fat absorptive patterns in the geriatric patient. *J. Am. Geriatrics Soc.*, 7: 941, 1959.
5. Mayer, J. and Yannoni, C. Z. Increased intestinal absorption of glucose in three forms of obesity in the mouse. *Am. J. Physiol.*, 185: 49, 1956.

DISCUSSION

Dr. Meyer Friedman (*San Francisco California*): When does the blood become completely free of radioactivity?

Dr. Berkowitz: In the normal subject, the blood radioactivity in twenty-four hours averaged about 2 per cent. In forty-eight hours, there was practically no radioactivity left.

Dr. Friedman: When did the blood radioactivity disappear completely in the most abnormal patient?

Dr. Berkowitz: Some maintained radioactivity for as long as seven days.

Dr. Friedman: Is the half-life about eight days?

Dr. Berkowitz: Yes.

Dr. W. Stanley Hartroft (*St. Louis, Missouri*): You said that obesity and coronary artery disease were associated and that more obese people have coronary artery disease than those who are not obese. There is evidence to question that, as I am sure you are aware. Actually, in autopsy data, where we have the full facts, we have not been able to obtain any correlation between weight and the incidence of coronary artery disease compared to control groups, either at time of death or as far back as we can go in the patient's history.

Have you been able to obtain any data of this type in your radioactive fat tests in diabetic and alcoholic patients or in those with cirrhosis?

It would also seem to me that there is evidence to suggest that the results obtained with this test would be affected by the state of caloric balance at the time. If the patients are gaining or losing weight during the time of this test, the results would be quite different. Were the patients who had had a coronary heart attack trying to lose weight, and do you have any data on the state of the fat balance? Also, have you discovered what are the results in this test when the quality of fat in the diet is varied, i.e., corn oil versus lard and so on?

DR. BERKOWITZ: We have not as yet had the opportunity to conduct such tests.

DR. J. POMERANZE (*New York, New York*): We have paralleled these tests a few years ago, but did not use radioactive substances. We used fat tolerance studies and our curves are exactly like those in obese patients with coronary artery disease.

We also tried to change the picture and succeeded in doing so by reducing the patients weight or eliminating fat from the diet at that time. Unfortunately, the technics for measuring fatty acids in the blood are difficult and we had to discard the test. However, this radioactive test achieves the same results in a simpler manner, and I believe it should be used in an attempt to treat these people by various means—use of a special diet and oils and lipotropics to see whether or not it has any effect on the absorptive curve.

DR. S. RODBARD (*Buffalo, New York*): Is it justifiable to draw the conclusion that because patterns of patients who have had coronary diseases are similar to those who are obese, one may say that the obese are "precoronaries" or "non-clinical coronaries?" Are these not separate conditions with similar patterns and not necessarily related to each other?

DR. POMERANZE: Obesity in itself is not a precursor of coronary artery disease, but I would guess that in a person who is predisposed to coronary artery disease, obesity will be a triggering mechanism. This is analogous to the obese patient with a predisposition to diabetes, in whom it will develop sooner if obesity is maintained. I would emphasize that the relation is possibly the same with coronary artery disease.

DR. CAMILLO ARTOM (*Winston-Salem, North Carolina*): This technic with iodized fat is certainly an interesting one. However, there may be some limitations, especially the possible liberation of iodine from fatty acid molecules.

When we used non-radioactive iodized fat, the amounts of iodine used had to be much higher to detect it. Large amounts of iodine were liberated, but it became evident that the whole molecule of fatty acid was not necessarily demolished. The possibility exists that the liberation of iodine occurred much before the fatty acid was demolished. For instance, when iodized fatty acids were administered, there was considerable fatty infiltration of the liver which did correspond to the amount of lipid iodine. Moreover, many of the fatty acids which were deposited in the liver had lost their iodine and were not detectable by the iodine technic. They were probably not natural and were changing their form. I don't know exactly what they were. They were not metabolized to the same extent as the natural fatty acids. That does not take away interest from this technic but implies an additional reservation about the interpretation of the results.

DR. BERKOWITZ: I think Dr. Rodbard's point is well taken. It may be that we have demonstrated similar metabolic alterations which have no more in common than that.

As to Dr. Hartroft's question, in the majority of diabetic patients we have studied, there has been an abnormal curve.

We were particularly interested in patients with cirrhosis. For a long time many have maintained that high fat diets are injurious to people with liver disease. We studied this and found normal patterns.

Relative to the state of nutrition of the patients in our studies, those with coronary disease were all of normal weight. None were attempting to lose weight. Our obese patients had been in that condition for a long time and this test was made soon after we saw them for the first time in a special obesity clinic.

DR. COLIN C. LUCAS (*Toronto, Canada*): My reaction may be colored by some experiences I had many years ago while working with Dr. Banting in a different field. We were interested in compounds analogous to the sulfa drugs. Most of you probably know that a compound almost indistinguishable chemically from sulfanilamide has no effect whatsoever on bacteria. If you move the 2-acid group from the para position to the meta position it is completely inactive. If you put a chlorine, a bromine or an iodine in either the para or the meta position of the OH group, the activity disappears completely; and if you put in a methyl or methoxy it would disappear completely.

That has always made me dubious about incorporating foreign additives. We often say that the amount being added is small compared to the total, but it is that small bit being measured, and whether or not it is any measure of the body's handling of the normal thing, I don't know. I wouldn't say, "Do not do it," but on the other hand I would be hesitant about drawing conclusions. You must have tools to work with and you must do the best you can with the tools. I am warning about the interpretation of the results.

DR. BACON F. CHOW (*Baltimore, Maryland*): I support the viewpoint of Dr. Lucas in that we want to make similar studies with radioactive vitamin B_{12}. We have studied tolerance curves produced by the intravenous injection of different amounts of cobalt^{60} labeled vitamin B_{12}. You can obtain different types of curves because part of the cyano group would be replaced by the hydroxy group. This type of study, while interesting clinically, should be interpreted carefully in terms of physiologic effects.

DR. BERKOWITZ: Dr. Spitzer, our biochemist, might like to comment on this.

DR. J. J. SPITZER (*Philadelphia, Pennsylvania*): It has been demonstrated that as far as absorption and transport are concerned—and I emphasize this as opposed to metabolism on the cellular level—there is good correlation between neutral fats and iodine-labeled neutral fats.

This does not hold true for metabolism on the cellular level, that iodine and the fatty acids part company; but absorption and transport through the body are the same.

DR. ARTOM: The difference has been pointed out at least in the rate of metabolism, and possibly also in the type of products first formed when the iodine is

taken away from the fatty acid molecule. In other words, there is probably a dehydration—by what mechanism I do not know, but it happens with brominated fatty acids. We have used brominated fatty acids and the bromine goes out even faster than the iodine, but this de-brominated fatty acid is still accumulating in the liver at a rate which is much higher than the normal fatty acid with hydrogen instead of iodine.

DR. B. A. SACHS (*New York, New York*): How are the total lipids extracted before measuring the radioactivity?

DR. BERKOWITZ: With trichloroacetic acid.

Lipoproteins and Lipotropes in Atherosclerosis

THADDEUS D. LABECKI, M.D.*

THE LIPOTROPIC substances are now recognized as one of the "legitimate" (if still experimental) treatments of atherosclerosis despite some dubious attitudes. For instance, Katz[1] now mentions lipotropic deficiency as one of the major contributing factors in the etiology of atherosclerosis.

In our considerations of atherosclerosis there are several pathologic processes which are frequently confused and considered as a single entity. It is questionable that the disease or lesion in a rabbit or any other experimental animal is comparable to the clinical entity which we term atherosclerosis in man.

Our experimental work in man is handicapped by the fact that we are forced to group our patients into two categories, the so-called "normals" and the "atherosclerotic individuals." There is no single test or even a group of tests which will allow us to divide our patients into two such categories.

One also frequently forgets the fact that a man may have a strategically located plaque and may die from coronary artery disease at the age of thirty-five years, while a person in whom atherosclerosis gradually develops may live to the age of seventy-five or eighty years and come to autopsy, at which time it is found that the cause of death is unrelated to the coronary artery disease.

Blumgart and Schlesinger[2] demonstrated by the injection technic that patients may have two coronary arteries completely occluded and still survive the ravages of coronary artery disease because adequate collateral circulation develops in them.

At times we get carried away in our enthusiasm and blame one factor alone for causing atherosclerosis. No one refutes this better than Katz in stressing the point that the disease, at least in man, is a multifaceted phenomenon, of which disturbed lipoprotein is only one factor.

The anatomy of the coronary tree is an extremely important factor, as proved by Enos.[3] The eddies and currents created by the tortuosity of the coronary tree are definitely a contributing factor. A second major question is: on what level do sex hormones act? We do not know whether it is on the level of permeability of the intima or of lipoprotein metabolism. Intravascular pressure is a third factor which must be considered and, in treatment and management of the disease, it must be properly controlled. Hypertension hastens the disease, probably as hypertension within the coronary system as well. All these factors must be kept in mind when we discuss the disease entity termed human atherosclerosis.

What do we have, consequently, as our therapeutic armamentarium against the human type of atherosclerosis? We cannot correct the anatomy of the coronary tree, just as we cannot, with any degree of great success, change our sex by administering hormones, even though they may be modified by their physiologic antagonists. This has been used experimentally by some investigators[4] but I do not think it offers hope of a practical treatment.

However, we can influence the lipoprotein metabolism. There are apparently few substances which can accomplish this. One of the classic substances is heparin, which hastens the production of the "clearing factor" (lipoprotein lipase). Treatment with heparin is impractical today for long term administration because of the cost and the inability to manufacture heparin for oral medication.

Lipotropic substances apparently do influence the lipoprotein metabolism. This may be

From the Heart Disease Control Unit, Mississippi State Board of Health, Jackson, Mississippi.

* Medical Consultant, Heart Disease Control Unit.

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proved with the aid of a modest test, the fasting-chylomicron index. This index can be affected by the administration of lipotropic substances. There is a definite group correlation, first reported by Zinn and Griffith[5] and which I later confirmed,[6] between the occurrence of clinically demonstrable coronary atherosclerosis and elevated levels of chylomicrons in the fasting state. Those levels can be lowered, as we reported in 1955.[7] This study has been repeated by Rawls[8] and confirmed.

Further interest was aroused recently by the unsaturated fatty acids. We studied the effect of unsaturated fatty acids in moderate doses (in comparison to the doses used by other investigators) because I questioned the advisability of administering large doses of either saturated or unsaturated fatty acids. I believe that we expose the intima to too much abuse if we administer large doses of even unsaturated fatty acids. Moderate doses of unsaturated fatty acids failed to lower cholesterol levels. There was a lowering in only six of twenty-five patients.

There was, however, a definite effect when those moderate doses of unsaturated fatty acids were combined with the lipotropic substances: beta lipoprotein was definitely and uniformly lowered by administration of a combination of the two.[9,10]

It is not known whether or not there is a true synergistic action between the unsaturated fatty acids and the lipotropic substances. Further studies, using similar technics, would be of great interest.

Although there is only one factor which we can apparently influence safely with a medicinal approach, the outlook as to the final outcome is optimistic. At this time 10 per cent of the total population has excellent collateral circulation. If we consider the human form of the disease as being an adaptive evolutionary phenomenon, then I believe the time element is important and through evolutionary processes our system of collateral circulation will eventually become 100 per cent effective.

SUMMARY

It is obvious that coronary atherosclerosis in the human being is an end result of a number of causative factors. In contrast, the laboratory-induced lesion in the animal is most often caused by a single experimental technic. Thus, there is no common ground for comparison. There is a distinct possibility that an absolute or relative lipotropic deficiency is behind the disturbed lipoprotein metabolism, one of the atherogenetic factors in the form of the disease found in man. It is possible to demonstrate objectively the effect of lipotropic substances on lipoprotein metabolism.

REFERENCES

1. Katz, L. N., Stamler, J. and Pick, R. Research approach to atherosclerosis. *J. A. M. A.*, 161: 536, 1956.
2. Blumgart, H. L., Schlesinger, M. J. and Davis, D. Studies on relation of clinical manifestations of angina pectoris, coronary thrombosis, and myocardial infarction to pathologic findings, with particular reference to significance of collateral circulation. *Am. Heart J.*, 19: 1, 1940.
3. Enos, W. F., Jr., Beyer, J. C. and Holmes, R. H. Pathogenesis of coronary disease in American soldiers killed in Korea. *J. A. M. A.*, 158: 912, 1955.
4. Stamler, J., Pick, R. and Katz, L. N. Experiences in assessing estrogen anti-atherogenesis in the chick, the rabbit, and man. *Ann. New York Acad. Sc.*, 64: 596, 1956.
5. Zinn, W. J. and Griffith, C. C. A study of serum fat globules in atherosclerotic and non-atherosclerotic male subjects. *Am. J. M. Sc.*, 220: 597, 1950.
6. Labecki, T. D. Hyperchylomicronemia and hypercholesterolemia: their correlation with clinical atherosclerosis. *Am. J. Clin. Nutrition*, 3: 132, 1955.
7. Labecki, T. D., Bright, I. B., Lake, W. W. and Thompson, C. C. Effect of a therapeutic regime on hyperchylomicronemia and hypercholesterolemia. *Am. J. Clin. Nutrition*, 3: 141, 1955.
8. Rawls, W. B. and Tichner, J. B. Lipid metabolism and the effect of a combined lipotropic agent. *J. Am. Geriatrics Soc.*, 4: 89, 1956.
9. Labecki, T. D., Bright, I. B., Lake, W. W. and Thompson, C. α and β lipoproteins and serum cholesterol levels following administration of unsaturated fatty acids. *Proc. Soc. Exper. Biol. & Med.*, 97: 260, 1958.
10. Labecki, T. D. Effect of lipotropic factors upon serum lipids and vascular disease in man. *Am. J. Clin. Nutrition*, 6: 325, 1958.

DISCUSSION

Dr. Meyer Friedman (*San Francisco, California*): I do not consider atherosclerosis in the rabbit the

equivalent of human atherosclerosis. The answer must be collateral circulation or new arteries inserted surgically. I do not believe we will ever reach a point curatively where we will just diffuse something.

I think we may prevent, but I do not think we can cure—certainly, I am sure not by lecithin or anything like it. This observation is supported by post mortem studies on coronary arteries. Second, I want to stress again the multiple etiology of this disease.

We agree regarding the effect of emotional stress in the production (not just the changes in lipoprotein metabolism) of cardiovascular disease. The evidence is so overwhelming that it cannot be ignored. It is surprising and totally discouraging as far as therapeutics are concerned.

DR. LABECKI: I agree with Dr. Friedman's last remark. We deplore the methodology existing at this time and our inability to measure, gauge and determine the effects of such strain on the production of blood vessel disease in man.

Dr. Friedman used the term "lecithin," implying that one of these lipotropic factors can melt away calcific or fibrotic tissue. Studies have proved such a claim to be untrue. The only claim now made is that those substances do exert definite measurable influence on lipoprotein metabolism, always keeping in mind the other factors which we cannot influence.

Until quite recently some texts never mentioned lipotropic substances. Then we did our study on the alcoholic patients in Mississippi.* We did not melt away the cirrhotic tissues in any magic manner. What we accomplished was a high rate of diminution of fatty infiltration following high intake of lipotropic substances.

However, the rate of diminution of the fatty infiltration with a high protein diet is definitely slower, as proved by biopsy studies of the liver.

DR. W. STANLEY HARTROFT (*St. Louis, Missouri*): Dr. Labecki's report is of great interest. Taking choline out of an animal's diet and putting it back has been instructive. However, all of us are somewhat hesitant about transferring our studies to man at this point.

You stated that hypertension was associated with a higher incidence of coronary arteriosclerosis. We do not believe there is any evidence to support this concept that hypertension is a factor in the production of coronary arterial disease. My colleagues, Drs. R. M. O'Neal and W. Thomas, correlated the weights of the kidneys at autopsy with blood pressure levels. There is a distinct inverse correlation: the higher the blood pressure, the smaller the kidneys. If an increase in the levels of blood pressure was etiologically important in myocardial infarction, the weights of the kidneys in patients dying from recent myocardial infarcts should be smaller than in any control series. Results of the studies by Drs. O'Neal and Thomas show no significant difference in the weights of kidneys of patients dying from myocardial infarction and a control series of patients dying from other causes. Therefore, it is hard to believe that hypertension is a factor in producing myocardial infarction.

The incidence of myocardial infarction in Negroes living in St. Louis, in New Orleans and in Washington, D. C., is one-fifth to one-tenth of the incidence of myocardial infarction of white persons living in these same centers; yet the incidence of hypertension among Negroes in these same three cities is much higher than in white persons of the same age, and hypertension in Negroes develops earlier in their lives than in white persons. Therefore, more Negroes are subjected to these elevated blood pressure levels for longer periods of time and yet myocardial infarction occurs less in this group than in white persons.

DR. JULIUS POMERANZE (*New York, New York*): In this age of rapid pharmacologic advance I would like to think that we could find a treatment for arterial trauma other than surgery. We can only treat it too late, when the damage is already done. There must be some way of reaching it before this stage if we recognize the fact that man is naturally a sclerotic animal and that we must do something long before the irreversible vascular calcification develops.

We have to think in terms of what causes acute myocardial infarction. There is an ulcer and then a thrombus forms, or there is bleeding. Sometime in the future I hope we will be able to reverse this thrombus formation or stop the bleeding.

Recent releases indicated that Dr. Ross of Parker Hospital has activated plasminogen to dissolve the plaque, and a group in Boston achieved the same result experimentally. Whether or not this is therapeutically feasible is not known at present, but I cannot feel that this is as weak a picture as would appear from the discussion here.

DR. HARTROFT: I think the picture is more optimistic than yours, Dr. Pomeranze. We know that we could reduce the incidence of myocardial infarction in this country almost to zero right now. During the war the incidence of myocardial infarction in England dropped to one-half of what it was in the previous six months to a year. This occurred also in Denmark during the war.

DR. CAMILLO ARTOM (*Winston-Salem, North Carolina*): And in Norway.

DR. HARTROFT: During the siege of Stalingrad the incidence of myocardial infarction in that city before the siege came to an end fell to zero. This fact has been confirmed by autopsy reports. The incidence of myocardial infarction rose again as soon as food was restored.

I think we are in a period with regard to heart disease comparable to diabetes before insulin was used. Diabetes could be completely cured in that era by starving the patient. We could probably do the same thing

* LABECKI, T. D. and BUSBY, C. L. Medicinal management of fatty alcoholic livers. In: A. M. A. Scientific Exhibits, 1955, p. 371. New York, 1956. Grune & Stratton.

with myocardial infarction. Therefore, even if calcification is present (and that would have been true in the European populations) you can still reduce myocardial infarction.

DR. POMERANZE: I do not think you would even have to starve your patients. The picture looks promising, I think.

DR. FRIEDMAN: I did not mean to be unduly pessimistic. I believe if the artery is left you can do a lot, and if there is infiltration you may be able to remove it. However, I do not agree with Dr. Hartroft about these countries. He has to explain why the incidence of hypertension diminished and why the suicide rate in Great Britain during its greatest siege increased.

I want to know what other things happened during the siege besides the lowering of the fat ration. Low intake of fat is not the only factor in these blockaded countries, unless you want to say that hypertension is due to a high fat intake.

DR. GEORGE F. WILGRAM (*Toronto, Canada*): In view of our own work, Dr. Lucas' statements and Dr. Hartroft's work, I feel fairly safe in stating that, to the best of my knowledge, there is not too much convincing evidence that lipotropic deficiency has something to do with human atherosclerosis.

There have been several reports in the literature to that effect. However, they either have been disproved or disclaimed. The work done by Dr. Hartroft and which I later continued indicates that the lipotropic deficiency observed in animals does not have any resemblance to the atherosclerosis of man.

How do you reconcile your findings with those presented here, namely, that a deficiency of lipotropic factors tends to decrease all classes of blood vessel disease and on the readministration of choline a rise to normal occurs.

I realize that Dr. Labecki is dealing with a different situation, giving choline to persons who already have enough choline. Yet, on physiologic grounds, it is hard to see on the basis of our findings how those two things go together. Would you mind elaborating on this point?

DR. LABECKI: I will be glad to. The curves shown given today were those of laboratory animals. I do not believe the metabolism of choline and the fate of lipotropic substances in human beings and in experimental animals is the same. There is a lot of evidence to support this.

DR. WILGRAM: There is a preponderance of evidence that many different species, including the monkey, are fairly close to the human being, and exhibit similar patterns. From a biologic viewpoint it is hard to understand why man should be so very different.

DR. LABECKI: By stating that lipotropic therapy is a recognized form of treatment of atherosclerosis I meant that it is recognized as a form of therapy for one of the contributing factors. You cannot disprove or disclaim records such as electrophoretic strips. That is how we determined the levels of beta-lipoprotein. The chylomicron index was lowered, but not in all the cases; one does not expect 100 per cent success. Dr. Rawls, who used exactly the same technic, confirmed our study and published his results about a year ago.

We are dealing here with group correlation which exists between the high cholesterol levels and coronary atherosclerosis and between the higher beta-lipoprotein levels and coronary atherosclerosis. Anything which would influence such elevated levels should be considered as a preventive rather than a therapeutic measure. Dr. Katz' studies on chicks on methionine-deficient diets are indeed very convincing.

DR. WILGRAM: You are dealing with choline and not with methionine. The methionine-deficient chick is so drastically different from a human being given extra choline that comparison or a common ground is not possible. A methionine-deficient chick is different even from a chick that has enough choline in the food and is given extra choline.

DR. LABECKI: Admittedly, the individual lipotropic substances administered are less effective. There is apparently some synergism which at this time we are unable to explain, probably because of the methodology.

DR. W. E. CORNATZER (*Grand Forks, North Dakota*): In reference to Dr. Wilgram's question about similarity in the rat and man, there is a similarity in the phospholipid synthesis as shown by giving a single dose of choline or methionine. They both stimulate phospholipid synthesis in the plasma following a given dose. The methionine can be given orally or intravenously. Chaikoff's studies with dogs gave identical results. Therefore, we can say that there is a similarity between man, the rat and the dog, in that phospholipid synthesis is affected by choline and methionine.

DR. LOUIS FREEDMAN (*New York, New York*): Dr. Wilgram, in reference to your interpretation of the resemblance of lesions in animals to those of man, are you referring to the application of some method of overcoming these lesions or is it an interpretation of the anatomic factors?

In your paper read at the New York Academy of Sciences (April 11, 1958) you referred to the vascular lesions in animals as the Mönckeberg type but stated that they have no direct or immediate clinical application.

However, you said they were useful in providing us with the know-how for the development of other methods to produce cardiovascular changes in rats. These newly-developed methods yield lesions that are indeed an appropriate experimental counterpart to atherosclerotic heart disease in man.

DR. WILGRAM: What I meant to say is that the lesions developed by choline-deficiency trained us in learning, understanding and know-how for the development of lesions by other methods. These "other" methods are definitely different from anything that has to do with choline deficiency.

The method described and used has many things in common with the approach that Dr. Hartroft has developed using a diet very rich in choline and other ingre-

dients which induces hyperlipemia and vascular anemia. Animals on a choline-deficient diet with cardiovascular disease have the opposite, namely, decreased blood lipid, as has been shown here by different and independent investigations.

The connection between choline deficiency and those coronary infarcts described is a personal one in our laboratory.

DR. COLIN C. LUCAS (*Montreal, Canada*): In other words, you wish to say that choline is good for curing choline-deficiency but not for curing other things?

DR. WILGRAM: That is my definite conclusion.

Effect of Soybean Phosphatides on Serum Lipids and Lipoproteins

BERNARD A. SACHS, M.D.*

OUR STUDIES on phospholipids and serum lipids were initiated following the observation that incubation of human serum with an emulsion of fats containing soybean phosphatides caused an increase in the migration velocity of serum lipoproteins. It appeared that the phosphatide used as an emulsifier was the active ingredient.

I plan to show that the soybean phosphatide complex produces this "lipoprotein shift" and that the inositol phosphatide fraction is responsible for this phenomenon.[1] The effect of the intravenous administration of this fraction on serum lipids and lipoproteins will also be demonstrated.

An extract of soybean phosphatide was dissolved in normal human serum. Aliquots of the mixture were streaked on filter paper strips for electrophoretic analysis immediately after mixing and after incubation at 37°C. for twenty-four hours. The strips showed a definite lipoprotein shift or increase in the migration velocity of the beta lipoprotein to the alpha. Addition of the soybean phosphatide to hyperlipemic serum produced a similar effect.

Whole soybean phosphatide complex consists of approximately equal quantities of lecithin, cephalin and inositol phosphatide, or lipositol. Fractionation with alcohol produces an alcohol-soluble lecithin fraction and an alcohol-insoluble lipositol fraction, both of which contain one-third cephalin. The alcohol-soluble fraction contains two-thirds lecithin and the alcohol-insoluble fraction contains two thirds lipositol.

Using these fractions and the same technic with normolipemic sera, we found that the alcohol-insoluble lipositol fraction had the same effect as the whole phosphatide complex, and the alcohol-soluble lecithin fraction had no effect on the migration velocity of the beta lipoprotein. Essentially the same results were obtained using the sera of patients with hyperlipemia.

The increased migration velocity of serum lipoprotein is similar to that seen after administration of heparin *in vivo*. The heparin effect is associated with the liberation of free fatty acids.[2] Incubation of either phosphatide fraction with human serum was associated with increases in free fatty acid. However, no difference between the two fractions was noted. This suggests that the lipoprotein shift is not due simply to the liberation of free fatty acids. Heating to 56°C. did not inhibit the reaction, but addition of adequate amounts of protamine *in vitro* did.

The *in vitro* studies with the lipositol fraction were repeated *in vivo* in twenty-one normal rabbits,[3] and an effect similar to that with heparin was obtained. In a half-hour there was a marked increase in migration velocity, which was still abnormal at the end of four hours; at the end of twenty-four hours it had returned to basically the normal or control value.

The serum lipid analysis, after intravenous administration of a 5 per cent emulsion of this alcohol-insoluble fraction, showed a marked increase in the total phospholipids at the end of one hour averaging 372 per cent, an increase in neutral fat of 124 per cent and in cholesterol of 93 per cent. To be more specific, there is a marked increase in phospholipids, a mobiliza-

From the Montefiore Hospital, New York, New York.

* Research Associate and Associate Attending Physician.

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tion of neutral fat and a lesser but significant elevation of cholesterol values in the normolipemic rabbit within three minutes after the phosphatide infusion. These values return to normal or almost normal after four hours and are slightly elevated after twenty-four hours. By the seventh day there is still a mild elevation in cholesterol and at the end of two weeks initial values are reached.

In the rabbits with hyperlipemia, following the phosphatide infusion, there is an increase in the phospholipid, neutral fat and cholesterol of the same magnitude as in the normal animal. However, the increase of neutral fat and cholesterol is delayed and the greatest mobilization of these lipids appears twenty-four hours after administration of the phosphatide. In experiments on rabbits with hypercholesteremia, using dextrose as the control, an unexplained fall in all lipids of similar magnitude is noted.

The soybean phosphatide is administered as an emulsion in 5 per cent dextrose. We believe that in the rabbits with hyperlipemia, the changes in lipids represent a summation effect of phosphatide and of dextrose.

These findings are of interest when evaluated with investigations of others. Lever and Waddell[4] noted falls in levels of serum cholesterol in hyperlipemic subjects following the infusions of lipid. These infusions were in 5 per cent dextrose, and the results obtained might be accounted for by the effects of dextrose alone. Gordon[5] and Dole[6] have shown that the rise in non-esterified fatty acids following fasting can be reversed following a glucose meal. Albrink et al.[7] recently demonstrated that the rise in triglycerides and nonesterified fatty acids following a fatty meal can be abolished by the administration of glucose. We are now using saline in addition to dextrose for a control.

To help explain the differences between the alcohol-soluble and the alcohol insoluble fractions, we have isolated lipositol from the soybean phosphatide and demonstrated that lipositol is responsible for the increase in the migration velocity of the serum lipoproteins.

We have given the lipositol fraction orally to ten patients with disorders of lipid metabolism. We noted no increased migration velocity of serum lipoproteins suggesting some alteration during digestion. Half of the patients showed significant falls in serum cholesterol levels.

SUMMARY

In vitro studies have shown that the alcohol insoluble inositol phosphatide (lipositol) fraction of soybean phosphatide complex produces an increase in the electrophoretic migration velocity of serum lipoproteins, while the alcohol soluble lecithin fraction is without effect. Lipositol isolated from the alcohol insoluble fraction was found to be the compound responsible for the observed effects. The *in vitro* effects were confirmed *in vivo* by the intravenous administration of lipositol fraction to normal rabbits. In the normal rabbit, the "lipoprotein shift" is accompanied by a prompt and profound mobilization of neutral fat and cholesterol to the blood stream. Oral administration of this fraction to patients failed to result in similar changes in serum lipoproteins but in half of those studied, significant falls in serum cholesterol levels were observed.

REFERENCES

1. Sachs, B. A. and Danielson, E. *In vitro* effect of soybean phosphatides on serum lipoproteins of normal and hyperlipemic subjects. *Proc. Soc. Exper. Biol. & Med.*, 93: 22, 1956.
2. Rosenberg, I. N. Serum lipids studied by electrophoresis on paper. *Proc. Soc. Exper. Biol. & Med.*, 80: 751, 1952.
3. Sachs, B. A., Danielson, E., Burack, B. and Leiter, L. *In vitro* and *in vivo* effects of inositol phosphatide (lipositol) fraction of soybean or serum lipids and lipoproteins. *J. Appl. Physiol.*, 13: 381, 1958.
4. Lever, W. F. and Waddell, W. R. Idiopathic hyperlipemia and primary hypercholesteremic xanthomatosis. VII. Effect of intravenously administered fat on the serum lipids. *J. Invest. Dermat.*, 25: 233, 1955.
5. Gordon, R. S., Jr. and Cherkes, A. Unesterified fatty acid in human plasma. *J. Clin. Invest.*, 35: 206, 1956.
6. Dole, V. P. A relation between non-esterified fatty acid in plasma and the metabolism of glucose. *J. Clin. Invest.*, 35: 150, 1956.
7. Albrink, M. J, Fitzgerald, J. R. and Man, E. B. Reduction of alimentary lipemia by glucose. *Metabolism*, 7: 162, 1957.

DISCUSSION

Dr. Meyer Friedman (*San Francisco, California*): I am glad to hear that Dr. Sachs is obtaining a hypercholesteremic effect with a phosphatide infusion. We

first started with either a glycerine preparation containing cephalin and the alcohol-soluble fraction, or normal saline. We now alternate these preparations with 5 per cent glucose or normal saline.

We were not able to induce hypertriglyceremia when using phosphatide during our experiments on rats. I am not sure that would be true in the rabbit which has a peculiar lipid metabolism and in which cholesterol, when elevated, remains high for a long period of time. In the rabbit hypertriglyceremia is not present after infusion of the type of phosphatides we used in our original study.

According to the experiments of Lever on human beings, the patients had a lower cholesterol level for several days after the infusion of lipid but when he used triglyceride or the phosphatide alone, he was unable to obtain the same results.

It is our belief that by perfusing the blood stream of the animal with triglycerides we are actually washing out surplus cholesterol from the various depots that are easily accessible to the blood. For a few days the animal will have a lower blood cholesterol level because he is not synthesizing it. If you use an animal with atherosclerosis which has its own deposits of triglyceride in the blood and introduce another triglyceride, i.e., sesame oil (which is very rapidly removed from the blood), you are competing in the blood for two areas of solubility. The cholesterol already present will go into the liver. Cholesterol can be removed from the animal with atherosclerosis by giving him fat, because this fat goes to the liver and is broken down into cholic acid. The cholesterol is removed from the blood by the liver, and the plasma cholesterol level, instead of being 800 mg. per cent for 24 to 48 hours will be 300 to 400 mg. per cent. In other words, it is practically an *in vitro* phase phenomenon These results may also be obtained with dextrose. When we perfuse the blood stream of the rat with dextrose there is, in general, a lower lipid level as determined by tissue analysis of cholesterol.

When we analyze the skin, liver or intestines six hours after an infusion of dextrose, there will be less, or about the same amount, of cholesterol in the tissues. A rise in the cholesterol level is never observed.

Infant-Feeding Practices and Blood Cholesterol Levels

JULIUS POMERANZE, M.D., WITH THE COLLABORATION OF ALTA GAULWIN, M.D. AND LAWRENCE B. SLOBODY, M.D.

THE RELATIONSHIP of lipid metabolism, serum cholesterol levels and atherosclerosis is still unresolved. The clinical importance of quantitative and qualitative changes in dietary fat intake also remains unresolved. However, it has been shown that unsaturated fats added to the diet in sufficient proportions will lower serum cholesterol levels in adults.[1] Many well-qualified observers believe that serum cholesterol levels serve as an important index of atherogenicity.

Holman's investigations[2] and those of other researchers over the world have indicated that atherogenesis is usually present during and after the third year of life. If this is true, and if serum cholesterol levels are important barometers of the degree of susceptibility to atherosclerotic changes, then lipid metabolism in infancy and childhood deserves intensive study. The present work was undertaken to determine serum lipid levels and the effect of various dietary changes on them in infants.

MATERIAL AND METHODS

At birth the serum cholesterol level of an infant averages 75 mg. per 100 ml. This level rises rapidly in an American child fed a formula of cow's milk. The major elevation in serum cholesterol levels takes place during the first few months of life.

During a serial study of serum cholesterol levels from birth, it was noted that in infants who had been fed a preparation of soy milk because of an allergic state the expected elevation of serum cholesterol did not occur. The soy milk preparation is high in essential fatty acids. The trend in these infants was similar to that described, in reports from Italy, where infants are usually breast-fed and early childhood diets contain large amounts of vegetable fats.

Corn oil contains 53 gm. of linoleic acid, 12 gm. of saturated fatty acids, 28 gm. of oleic acid and 1.5 gm. of sitosterol per 100 gm. Therefore 50 gm. of corn oil were added to the evaporated milk formula in a homogenous mixture obtained with a food blender. This formula was alternated with one of plain evaporated milk. Serum cholesterol levels were determined at weekly intervals from blood drawn from the femoral vein. The study was continued until an infant was discharged from the hospital because of parental or guardian demand.

RESULTS

The results demonstrated in these experiments with infants parallel those of similar investigations in adults. There was a depressant effect of corn oil, with its large amounts of essential fatty acids, on serum cholesterol levels.[3] Breast milk also contains a high percentage of essential fatty acid (53 per cent unsaturated fatty acids, 47 per cent saturated fatty acids) and should, therefore, depress serum cholesterol levels. Breast milk also differs from evaporated milk in protein and total fat content (1.2 per cent protein in breast milk and 2.2 per cent protein in evaporated milk diluted 1:2). It might be expected that in infants fed breast milk the lipid findings would be similar. This has not been found true.

From the New York Medical College, Metropolitan Medical Center, New York, New York.

Presented at the First Symposium of the Lipotropic Research Foundation, April 13, 1958, Philadelphia, Pennsylvania.

This study was continued during the year following the experiment described. Serum cholesterol levels were determined monthly from birth, in infants fed an evaporated milk formula. Similarly, cholesterol levels were determined in infants fed a prepared milk formula* containing 55.9 per cent of total fatty acids as unsaturated fats. (Cow's milk fat is 43 per cent unsaturated.)

Fifty-three normal infants fed an evaporated milk formula and twenty-seven normal infants fed a prepared milk formula have been observed for periods up to four months. The serum cholesterol levels differ in these two groups. The average cholesterol levels for the infants fed the evaporated milk formula were: after two weeks, 98 mg. per 100 ml.; after four weeks, 128 mg. per 100 ml.; after eight weeks, 162 mg. per 100 ml.; after twelve weeks, 187 mg. per 100 ml. The latter level appears, from our data, to persist for a number of years through childhood. The infants fed prepared milk had lower serum cholesterol levels: after two weeks, 84 mg. per 100 ml.; after four weeks, 98 mg. per 100 ml.; after eight weeks, 123 mg. per 100 ml.; after twelve weeks, 128 mg. per 100 ml. At sixteen and twenty weeks, serum cholesterol levels of both groups began to approximate each other. During this period cereal, whole milk, meat and dairy products were added. Those infants receiving no supplement to the formula except fruit did not experience a rise in serum cholesterol levels.

COMMENTS

Limited data on breast-fed infants do not appear to be in agreement with that found in the foreign literature. Serum cholesterol levels in these infants are considerably higher than would be expected from a milk relatively high in unsaturated fat. Maternal diets have been shown to qualitatively alter the fatty acid content of breast milk. American mothers have high caloric and high saturated fat intake. The difference in maternal diets may explain the discrepancy between our findings and those reported in the foreign literature. Serum cholesterol levels must be studied in breast-fed infants of mothers receiving varying fat diets.

The atherogenetic process may be reversible during the formative years. Therefore, if nutrition affects atherogenesis, it must be a constant influence over the life span of the individual. It is possible that many nutritional aspects of infancy and childhood play prominent roles in the physiology and pathology of adulthood, not only by virture of habits learned but also by metabolic alterations which are maintained.

Present day efforts are concerned with reversing the atherosclerotic changes encountered in adulthood and averting maligant sequelae. Preventative medicine would be better served however, if alterations in the etiologic factors concerned with atherosclerosis were attempted during the formative years when significant remissions are more easily accomplished. If dietary changes are to be made, these changes would be most useful if begun during early life.

In this experiment with infants and in others in adults, supplemental feeding of essential fatty acid presents a serious problem of the added calories. Fifty grams of corn oil increases the total daily caloric intake of these infants considerably. It is difficult to construct a program to lower serum cholesterol without creating or aggravating the problem of obesity. Infants fed this additional formula gain weight more rapidly. The prepared milk formula used in the present experiment eliminates this caloric excess.

If a preventative program in infancy were to be undertaken, it should be considered that encouraging the development of larger babies and probably larger adults by added calories could adversely affect our antidegeneration program by placing a greater burden on unadapted organs and biochemical mechanisms. Caloric excess can be avoided by substitution of essential fatty acid for saturated fats present in milk.

A high intake of essential fatty acid may lower serum cholesterol levels in infants. This does not make the link between lipidemia

* S.M.A., supplied by Wyeth Laboratories, Inc., Philadelphia, Pennsylvania.

and atherosclerosis clearer. Until a method more objective than biochemical is available for measurement of atherosclerosis in man, epidemiologic studies will continue to give us inconclusive and conflicting answers to this important problem.

REFERENCES

1. Ahrens, A. H., Jr., Hirsch, J., Insull, W., Tsaltas, T. T., Blomstrand, R. and Peterson, M. L. Dietary control of serum lipids in relation to atherosclerosis. *J.A.M.A.*, 164: 1905, 1957.
2. Holman, R. L. The lipids in relation to atherosclerosis. *Am. J. Clin. Nutrition*, 8: 95, 1960.
3. Pomeranze, J., Goalwin, A. and Slobody, L. B. The effect of a corn oil—evaporated milk mixture on serum cholesterol levels in infancy. *Am. J. Dis. Child.*, 95: 622, 1958.

DISCUSSION

Dr. W. Stanley Hartroft (*St. Louis, Missouri*): How much corn oil did you give the adults?

Dr. Pomeranze: We gave them 6 ounces a couple of times a day—very large doses. These were cooperative patients and we found a significant lowering of serum cholesterol levels.

Dr. Hartroft: That is nearly 1,500 or 2,000 calories.

Dr. Pomeranze: Yes. Whether or not they took all of it, I do not know. Again, what the significance of all of this is, I have no way of knowing. However, I would suggest that if we are going to attack this problem from a dietary point of view, infancy is the time to attempt it.

Dr. R. Alfin-Slater (*Los Angeles, California*): Serum cholesterol levels always worry me when they are unaccompanied by any other determination. Did you perform any fecal sterile analysis on the babies at all?

Dr. Pomeranze: No.

Dr. Alfin-Slater: What happens to the cholesterol that is removed from the blood?

Dr. Pomeranze: I have no idea.

Dr. Alfin-Slater: Do you not think it would be helpful to make such an analysis?

Dr. Pomeranze: It surely would.

Dr. W. E. Cornatzer (*Grand Forks, North Dakota*): Professor Joseph Anderson from the Physiological Hygiene Laboratory of the University of Minnesota, recently described a study made there on 180 medical students. Two different laboratories performed the cholesterol determinations in this group who took the corn oil daily for four weeks in the form of a milkshake. There was a slight gain in weight in many of the students and a drop in the blood cholesterol levels.

Dr. Pomeranze: I am not offering our findings as conclusive. I am not convinced of the importance of cholesterol levels as a barometer of atherosclerosis, but I think that it is as good a biochemical instrument as we have for the type of work going on at this time.

Dr. Alfin-Slater: Are there any figures available on cholesterol levels in babies fed breast milk as compared with those fed a formula?

Dr. Pomeranze: I have gone through the available literature and there are figures from Italy and from one other country where the babies were breast-fed; and in those babies the cholesterol levels were kept fairly low. They paralleled our figures with the soybean preparation.

SUMMARY OF SYMPOSIUM

Dr. C. Artom (*Winston-Salem, North Carolina*): Dr. Hartroft, would you sum up the facts and point out the areas for further investigation?

Dr. Hartroft: I think this meeting has served a useful purpose in bringing together not only current data from recently performed experiments but also some of the earlier work which we tried to collate with newer knowledge. Dr. Artom and Dr. Cornatzer pointed out in a broad way the two possible sites of the mechanism and action of choline. It may act both in the tissues and in lipid transport.

It is over a quarter of a century since the lipotropic factors were discovered and named in 1932 by Best and his co-workers. In that first paper the possibilities of where and how choline acts were pointed out. We are still debating these same possibilities twenty-five years later, albeit with new information, but still without definitive answers. This area is one urgently requiring further investigation.

Dr. Lucas, speaking on the transport of cholesterol, added new data that are very exciting, clearly showing the effect of choline on the serum cholesterol in the presence of exogenous cholesterol.

There are certainly some obvious questions raised here. Where does the cholesterol come from when it is increased in the serum in this manner? Where does it go when the choline is removed and the serum cholesterol level decreases? One explanation could be intestinal absorption but I gather this explanation had been eliminated.

Dr. C. C. Lucas (*Toronto, Canada*): I think it is ruled out, of course, where there is no cholesterol being fed; but when cholesterol is fed I do not know how else to explain what we see, other than the effect of absorption, but there may be some more subtle thing.

Dr. Hartroft: You have not analyzed the fecal excretion of cholesterol?

Dr. Lucas: No.

Dr. Hartroft: Dr. Chow has emphasized an important factor—the background composition of the diet in regard to calories and absorption of food and vitamin content are important in interpreting any results in clinical investigation as well as with animals.

Dr. Berkowitz' technic on radioactive iodine absorption and radioactive fat absorption is interesting. There is a little reservation as to how widely this method should be applied in patients until more observations have been made.

Dr. Moon in California and others have now shown that lipid plaques may be present in newborn infants, indicating that the disease can begin *in utero*. There is growing evidence that a number of the so-called "degenerative" diseases that manifest their symptoms in old age may have had beginnings much earlier in life.

DR. LOUIS FREEDMAN (*New York, New York*): Perhaps the data from the laboratory and clinical and animal experimentation are not so controversial as is the interpretation. Similar data are interpreted in different ways, depending on whether it is applied to animals actually used or to man. Perhaps more careful use of statistical evaluation of the data is needed and more appreciation that data on animals may have some application to man if properly interpreted.

Editorial

The Late Effects of Early Nutrition

NUTRITIONAL research has passed its pioneering period, when it was relatively easy to find, indeed, almost stumble upon "gold" in the form of new nutrients or of unsuspected nutritional effects by known food constituents. Vitamins, essential amino acids, unsaturated fatty acids and trace elements come under this category. Nevertheless, it would be fallacious, although stated recently, to assume that nutritional research has reached its zenith and has entered a more static state, or even a downgrade course. As a matter of fact, there are indications in several directions of vigorous new advances in the recognition of nutrition as an environmental factor of overriding importance.

Genetics has long been claimed to be *the* determining factor in growth and development: environmental factors, such as nutrition or infections, were credited only with transient effect. More recently observations are accumulating both from experimental and purely clinical studies which may cast some doubt on the rigid division between lasting genetic and temporary nutritional effects.

The most impressive example of a lasting and apparently fixed effect of nutritional deficiency on growth and development has been furnished through experimental studies on rats in the laboratory of R. A. McCance in Cambridge, England.[1]

Several newborn litters of rats of identical genetic strain were mixed and divided among the lactating dams, only a few to some and large numbers to others. After weaning (for the same duration) all rats were put on an identical commercial ration. Those rats which were nursed in large numbers (up to twelve) by one mother, were small at the time of weaning; the control animals which were kept with the mother in small number (about four) were large. This difference in size was not corrected by postweaning *ad libitum* feeding of a regular commercial ration; the animals originally underfed remained small throughout the extended period of observation. Whether this late effect was solely due to the original caloric deficiency or to some other more qualitative deficiency must remain unanswered at the present time.

These experimental observations have their counterpart (although not as well controlled and, in fact, open to some criticism) in the statistical data obtained during the last few years on Japanese children. The small stature, thinness, lower average body weight of the Japanese have long been attributed to racial, inherent qualities. Recent anthropologic measurements, investigations on the nutritional state and dietary surveys seem to support the interplay of environmental factors, chiefly nutritional for the anthropologic constants of Japanese children and adults rather than that of purely genetic influences. Even first generation Nisei children in America approach the average American children in their growth and development.[2] In Japan during the last war there was a severe reduction in length and weight of children. Since the war general improvement has set in, with a slow but continuous increase in the figures for body length and weight. In the rural population growth and development still fall significantly behind that of the urban population. This is best correlated with general caloric undernutrition and to some extent perhaps also with a low fat intake.[3–5] The same conclusion may be drawn from the diet of Japanese infants after weaning, based on the detailed data on food intake in the postweaning period collected and published recently in Japan by a special Committee under the chairmanship of Professor Enjoji of Kyushu University.[6]

Admittedly, these statistical observations on

infants, children and adults in Japan and on Japanese stock in North America could be the results of continuous (nutritional) effect and not necessarily the sequelae of early nutritional deficiency, as in the aforementioned rat experiments. Nevertheless they bring to the fore the importance and interaction of exogenous factors including nutrition.

The specific after-effect of early dietary deficiency on the development of particular pathologic conditions is well documented by the interesting experimental studies of Hartroft and Best[7] who have found severe hypertension, developing late in life, in a group of rats kept only one week in early life on a diet deficient in choline. How far the promotion of degenerative diseases and malignancy seen in rats fed a high calorie diet and, at least under special conditions, the enhancing effect of protein on the same group of diseases[8] should be considered as sequelae of an early harmful influence or as a continuous process, is still an open question. These experiments on animals may have no bearing on human nutrition but at least they make long extended follow-up studies on the conceivable late consequence of infant feeding in its present form desirable. For pediatricians for example, it would be of special interest to know more about the possible late effects of breast milk versus "artificial" feeding.

This field, the late effects of early nutrition, encompassing the whole life span of animals and man, is worthy of intensive exploration and should yield important new knowledge.

PAUL GYÖRGY, M.D.
Philadelphia General Hospital
Philadelphia, Pennsylvania

REFERENCES

1. WIDDOWSON, E. Personal communication.
2. GREULICH, W. W. Growth of children of the same race under different environmental conditions. *Science*, 127: 515, 1958.
3. Malnutrition and Nutrition Activities in Japan. Ministry of Health and Welfare. Japanese Government, 1956.
4. OHMORI, K. The relation between nutrition and physical constitution. *Keio J. Med.*, 7: 157, 1958.
5. OISO, T. The nutritional status of the Japanese people and its future prospect. *Japanese M. A.*, April, 1959.
6. ENJOJI, M. Report of the Committee on Weaning, 1958.
7. HARTROFT, W. S. and BEST, C. H. Hypertension of renal origin in rats following less than one week of choline deficiency in early life. *Brit. M. J.*, 1: 423, 1949.
8. ROSS, M. Protein, calories and life expectancy. *Fed. Proc.*, 18: 1190, 1959.

Clinical Reports

Nutritional Status of Adolescent Children

IV. Cholesterol Relationships*

MARGARET MCGREGOR HARD, M.S.† AND NETTIE C. ESSELBAUGH, PH.D.‡

CHOLESTEROL metabolism and the fate of food cholesterol in the human organism are of considerable interest to nutritional and medical personnel. Okey[1] has stated that those factors likely to be involved in cholesterol metabolism are the amount of dietary cholesterol; the amount and composition of the fat, phospholipids and protein in the diet; the amount and type of dietary lipotropic agents and the status of the person as to hormone secretion and vitamin intake.

As part of a study of the nutritional status of adolescent children[2–4] information was obtained on cholesterol and vitamin intake, serum cholesterol level, intake of animal and vegetable fat and protein, basal metabolic rate, weight, hemoglobin, hematocrit and blood pressure. Relationships between cholesterol and these factors in a normal adolescent group consuming an average American diet were examined and are reported herein.

PROCEDURE

The subjects for the study were 248 fifteen and sixteen year old boys and girls who had been born and reared in either Snohomish or Yakima County of Washington. Approximately equal numbers of boys and girls made up each age group in each area.

Dietary Data

The amounts of intake of cholesterol, fat, protein and vitamins were obtained from semi-quantitative seven-day dietary records.[2] Vitamin supplements were recorded separately. Cholesterol content of the diets was calculated from values supplied by Okey[5,6] supplemented by information from Dobbin, Gofman, Jones, Lyon and Young.[7] The other nutrients were calculated from values in the U.S. Department of Agriculture, Agriculture Handbook Number 8,[8] Bowes and Church,[9] Bradley,[10] and Boyd, Eads and Sandstead.[11]

Serum Cholesterol

Free and total cholesterol determinations were made on fasting blood. Serum was stored in the frozen state until it could be analyzed by the method of Schoenheimer and Sperry.[12]

Physical Measurements

Basal metabolic rates were determined in duplicate with the Sanborn metabolator. The subjects slept at the laboratory the night previous to the determination. Weight and blood pressure were taken as part of an extensive physical examination performed by a research physician. Hemoglobin was determined by Wintrobe's alkaline hematin colorimetric method,[13] and hematocrit by the Wintrobe and Handsberg method.[13]

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† Professor of Food and Nutrition, Washington State University, Pullman, Washington.

‡ Present address: Sumner, Washington.

TABLE I
Mean Daily Intake of Cholesterol, Calories, Protein and Fat by Area and Sex

Nutrient	Snohomish County		Yakima County	
	Boys (63)	Girls (62)	Boys (61)	Girls (61)
Cholesterol (mg.)	837.0 ± 35.0*	579.0 ± 23.0*	782.0 ± 30.0*	575.0 ± 24 0*
Calories	3,181.0 ± 90.13	2,213.0 ± 67.13	3,119 0 ± 67 65	2,048.0 ± 57.86
Protein				
Total (gm.)	108.9 ± 3.22	75.9 ± 2.54	101.2 ± 2.60	71.8 ± 2.76
Per cent of calories	14.0	14.0	13.0	14.0
Animal (gm.)	71.1	51.4	63.7	48.5
Vegetable (gm.)	37.9	24.5	37.5	23.4
Fat				
Total (gm.)	146.2 ± 5.53	100.2 ± 3.82	141.3 ± 4.68	91.9 ± 3.40
Per cent of calories	41.0	40.0	40.0	40.0
Animal (gm.)	94.8	63.8	84.4	56.2
Vegetable (gm.)	51.4	36.5	56.9	35.7

* Standard error of the mean.

Statistical Treatment of Data

Daily mean intake of cholesterol, animal and vegetable protein and fat, total fat, total protein and calories were obtained for boys and girls in Snohomish and Yakima counties. Free and total serum cholesterol, body weight, hemoglobin, hematocrit, systolic and diastolic blood pressures, and basal metabolic rates also were expressed as mean values for these groups of subjects.

Analysis of variance was used for comparison between groups.[14]

Correlations were calculated for the four groups. Cholesterol intake was correlated with serum cholesterol level, weight, and basal metabolic rate. Serum cholesterol was correlated with fat, protein, calorie and vitamin intake, basal metabolic rate, weight, hemoglobin, hematocrit and blood pressure.[14]

RESULTS AND DISCUSSION

Cholesterol Intake

Analyses of variance revealed a significant difference ($P < 0.01$) between the cholesterol intake of the boys and girls (Table I). There were no significant differences ($P > 0.05$) between areas or between the fifteen and sixteen year old subjects. The intake of subjects from Washington was similar to that of the adolescents from Idaho.[15] Subjects from both Washington and Idaho consumed somewhat larger amounts of cholesterol than the thirteen to fifteen year old subjects in Colorado, Montana, or Utah[15] (Table II).

On the whole, the cholesterol intake of the teen-age groups is similar to values reported for younger and older age groups (Table II).

Relation to Weight and Basal Metabolic Rates: Cholesterol intake was not significantly ($P > 0.05$) correlated with weight or with basal metabolic rate. This was true for both boys and girls. Separation of the subjects into those that were 20 per cent or more overweight or underweight did not change the correlation.

Serum Cholesterol Levels

Significant differences ($P < 0.01$) in total serum cholesterol were found between areas and between sexes. The subjects from Yakima (boys and girls combined) had higher values than those from Snohomish. The serum levels were consistently higher in girls (both areas combined) than in boys (Table III). About 25 per cent of the total cholesterol was in the free form in all groups. These values are similar to serum cholesterol values reported for subjects in Idaho[16] (Table III). They are, however, higher than values given for teen-agers from Montana[17] and for thirteen to nineteen year old subjects from Utah.[18]

TABLE II

Comparison of Cholesterol Intakes and Serum Cholesterol Levels for Subjects of Varying Ages

Location	Age (yr.)	Cholesterol Intake (mg./day)			Total Serum Cholesterol Levels (mg./100 ml.)		
		Reference	Male	Female	Reference	Male	Female
New York	2 mo. to 3 yr.	..	..	..	20	171.4	179.2
	4, 5 and 6	..	..	..	..	175.7	179.9
	7, 8 and 9	..	..	..	..	185.8	192.7
Utah	5 to 9	15	544	496	18	159.0	175.0
New York	10 to 12	..	..	..	20	189.1	180.9
Utah	10 to 12	15	629	588	18	176.0	176.0
Utah	13 to 19	15	744	519	18	154.0	162.0
Colorado	13 to 15	15	593	418	..	...	...
Montana	13 to 15	15	783	471	17	133.0	144.0
Idaho	13 to 15	15	804	523	16	166.3	175.3
Washington	15 to 16	..	810	577	..	165.3	177.6
Utah	25 to 49	15	932	545	..	...	...
California	50 to 54	15	772	480	19	252.0	267.0
Colorado	50 to 54	15	644	379	..	...	...
California	80+	15	624	349	19	216.0	236.0
Colorado	80+	15	534	349	..	...	...

The difference between areas is difficult to explain. Dietary intake of cholesterol, protein, fat and calories (Table I) tends to be lower in Yakima than in Snohomish county although the differences are not significant ($P > 0.05$). Two other factors may have a bearing, e.g., the difference in the amount of sunshine in Snohomish and Yakima counties, and the difference in the physical build of the boys in the two areas. The Yakima area, on the average, has approximately twice as much sunshine as the Snohomish area.[2] The reconversion of 7-dehydrocholesterol formed by the action of the sun on skin cholesterol might account for some of the increased cholesterol in the serum of the subjects from Yakima. Kritchensky[19] points out that 7-dehydrocholesterol can be converted to cholesterol. Another contributing factor may be body build. Kornerup[20] reports that slender men have lower serum cholesterol levels than heavy men. Twenty-eight of the Snohomish boys (44 per cent) and fourteen of the Everett boys (23 per cent) could be classified as "slender." The average serum cholesterol value of the slender Snohomish boys was 150 mg./100 ml. but 179 mg./100 ml. for the slender Yakima boys. The additional number of slender boys in the Snohomish group may account for some of the area difference in serum cholesterol levels.

Differences in serum cholesterol levels between the sexes have been found in adolescents in Montana[17] and Idaho[16] and in elderly subjects in California.[21] There appears to be a tendency for the serum level of prepuberty girls to be somewhat higher than the level of the boys (Table II) although the difference is of doubtful significance. During adolescence, the level appears to drop, particularly in the boys. A variation in hormonal control is suggested although this may not be the only factor involved. Aftergood, Deuel and Alfin-Slater[23] have postulated that the increased cholesterol plasma level in female rats is due to the smaller requirement of the female rat for essential fatty acids. This, in turn, releases more of the ingested essential fatty acids for transport of cholesterol. Although the human requirement for essential fatty acids is not well established, this hypothesis may partially explain the sex differences observed in human serum cholesterol levels.

Effect of Dietary Constituents: A significant correlation did not exist between cholesterol intake and total serum cholesterol ($r = -$

TABLE III
Mean Serum Cholesterol Values by Area and Sex

Total Serum Cholesterol Levels	Snohomish County		Yakima County	
	Boys (63)	Girls (61)	Boys (62)	Girls (61)
Mean (mg./100 ml.)	156.7	173.7	173.9	181.4
Range (mg./100 ml.)	98.8–232.6	115.1–297.9	111.0–244.7	123.0–235.1
Standard error	4.17	4.81	4.13	3.62
Free (% of total)	24.6	25.5	26.2	26.4

0.1853 for the girls; r = + 0.1813 for the boys). Cholesterol intake and free serum cholesterol did correlate significantly for the subjects from Snohomish (r = − 0.5187 for the girls; r = + 0.2697 for the boys). The negative correlation for the girls and the positive correlation for the boys emphasizes the sex-linked difference: the cholesterol intake of the girls was lower than that of the boys but the serum level was higher (Table II). An association between free serum cholesterol and cholesterol intake was found also in teen-age subjects from Montana.[17] A positive correlation between total serum cholesterol and daily cholesterol intake of aging subjects was reported from California and Wyoming.[21,24]

Significant correlations ($P<0.05$) with calories and with protein intake occurred only with the boys from Snohomish: calories correlated positively ($P < 0.05$) with free and total serum cholesterol; protein correlated ($P < 0.01$) with free and total. The proportion of animal to vegetable protein was similar for all groups, ranging from 62.9 per cent to 67.8 per cent animal protein and from 32.2 per cent to 37.1 per cent plant protein. No significant relation ($P > 0.05$) was found between the serum cholesterol level and fat intake. The proportion of animal to vegetable fat ranged from 59.7 per cent to 64.9 per cent animal fat and from 35.1 per cent to 40.3 per cent plant fat (Table I). An examination of Tables I and III indicates a lack of positive association between the serum cholesterol level and the intake of protein, fat and calories. The girls from Yakima with the highest level of total serum cholesterol consumed the smallest amounts of fat, protein and calories. Conversely, the boys from Snohomish with the highest consumption of cholesterol, calories, protein and fat, had the lowest level of total serum cholesterol. Guild and Odland[17] confirm these findings in teen-agers. In aging Californian subjects[21] protein and fat intakes did relate significantly to serum cholesterol values. Swanson et al.,[25] studying midwestern women ranging in age from twenty to ninety years, reported that dietary protein and fat explained a negligible amount of the variation in serum cholesterol.

The intakes of some of the vitamins correlated positively with serum cholesterol levels in the boys from Snohomish: thiamine with free and total ($P < 0.05$), riboflavin with free and total ($P < 0.01$); niacin with free and total ($P < 0.05$); vitamin A with free and total ($P < 0.05$); and ascorbic acid with free and total ($P < 0.01$).

Positive relationships were also noted in the boys from Snohomish between calcium intake and total serum cholesterol ($P < 0.05$), calcium and free ($P < 0.01$), phosphorus intake with free and total ($P < 0.01$), and iron intake with free and total serum cholesterol ($P < 0.01$). It is difficult to explain the vitamin and mineral correlations. They may have occurred merely through chance. There is always the possibility, however, of a metabolic relationship as yet not identified. For instance, serum ascorbic acid as well as ascorbic acid intake correlated ($P < 0.05$) with free and total serum cholesterol. Also a significant positive correlation ($P < 0.01$) was found for all subjects between serum carotene and free and total serum cholesterol. A possible relationship between ascorbic acid and cholesterol

TABLE IV
Mean Values for Selected Physical Measurements

Physical Measurement	Snohomish County				Yakima County			
	No.	Boys	No.	Girls	No.	Boys	No.	Girls
Body weight (lb.)	63	135.0 ± 2.84*	62	117.0 ± 2.05*	61	135.0 ± 3.67*	61	121.0 ± 2.12*
Basal metabolism (cal./sq. M./hr.)	62	38.9 ± 0.488	60	34.0 ± 0.498	61	39.8 ± 0.427	61	33.8 ± 0.408
Hemoglobin (gm./100 ml.)	63	14.6 ± 0.098	62	13.6 ± 0.093	61	15.0 ± 0.143	62	13.3 ± 0.149
Hematocrit (%)	63	48.5 ± 0.331	62	45.2 ± 0.294	61	47.2 ± 0.321	62	43.8 ± 0.263
Blood pressure	63		60		61		61	
Systolic (mm. Hg)	..	115.0 ± 1.27	..	109.0 ± 1.16	..	115.0 ± 1.37	..	108.0 ± 1.04
Diastolic (mm. Hg)	..	70.0 ± 1.06	..	67.0 ± 0.91	..	70.0 ± 1.00	..	69.0 ± 0.86

* Standard error of the mean.

and between carotene and cholesterol is suggested. Ascorbic acid and cholesterol metabolism is currently under further investigation in this laboratory.

Relation to Body Weight: Mean body weights for the four groups of subjects are given in Table IV. A simple correlation between weight and serum cholesterol revealed no relationship between these two factors for the groups as a whole. Likewise, there was no evident relationship between body weight and total serum cholesterol in subjects who were 20 per cent or more overweight or underweight. This contrasts with findings in Montana[17] where free and total serum cholesterol levels were positively associated with weight deviation in fifteen year old boys. In aging subjects in California[21] extreme under- or overweight was found to be associated with low and high serum cholesterol levels in men but not in women. Also, Payne[24] found no relation between weight and serum cholesterol in aging women. Swanson et al.[25] reported no significant relationship between weight and serum cholesterol in women from twenty to ninety years of age.

Relation to Basal Metabolic Rate: The mean basal metabolic rates for each of the four groups of subjects are presented in Table IV. These values are within the range given for adolescents.[26] Serum cholesterol and basal rates have been closely associated in hypothyroidism.[27] However, in these subjects a positive significant correlation ($P < 0.05$) was found only with the boys from Snohomish ($r = + 0.3143$). This finding is similar to results found in healthy subjects in the midwestern states.[21,25]

Relation to Blood Pressure: No relation was found between blood pressure and total serum cholesterol. Systolic and diastolic pressures (Table IV) were within the normal range for fifteen and sixteen year old boys and girls[26,29] Gillum et al. and Swanson et al.[21,25] also found no relationship between blood pressure and serum cholesterol levels in men and women.

Relation to Hemoglobin and Hematocrit: Payne[24] has recently reported a significant correlation between hemoglobin and total serum cholesterol in women over sixty years of age living at an altitude of 7,200 feet. This relationship, she postulates, may be due to the high altitude and to hematopoietic changes occurring with age. Similar correlations for the adolescent age group have not been reported to date.[16,17,30] In the adolescent subjects in Washington, hemoglobin and hematocrit positively correlated ($P < 0.05$) with free and total serum cholesterol in the girls from Snohomish. Hemoglobin and hematocrit values of these subjects (Table IV) are similar to those reported for adolescent girls from Idaho, Montana and Utah.[16,17,30]

SUMMARY AND CONCLUSIONS

Relationships between the serum cholesterol content and food intake (cholesterol, protein.

fat, vitamins, calories), body weight, basal metabolic rate, blood pressure, hemoglobin and hematocrit of 248 adolescent boys and girls are examined.

The aforementioned factors which have been reported to influence cholesterol metabolism in some other groups appear to have little if any relationship to serum cholesterol content in healthy fifteen and sixteen year old subjects consuming a normal American diet. The effect of sex on total serum cholesterol level is definite. This suggests that hormone secretion is one of the main factors regulating cholesterol metabolism during the adolescent period.

REFERENCES

1. OKEY, R. Use of food cholesterol in the animal body. Relation of other dietary constituents. *J. Am. Dietet. A.*, 30: 231, 1954.
2. HARD, M. M. and ESSELBAUGH, N. C. Nutritional status of selected adolescent children. I. Description of subjects and dietary findings. *Am. J. Clin. Nutrition*, 4: 261, 1956.
3. DONALD, E. A., ESSELBAUGH, N. C. and HARD, M. M. Nutritional status of selected adolescent children. II. Vitamin A nutrition assessed by dietary intake and serum levels. Biomicroscopic and gross observations. *Am. J. Clin. Nutrition*, 6: 126, 1958.
4. HARD, M. M., ESSELBAUGH, N. C. and DONALD, E. A. Nutritional status of selected adolescent children. III. Ascorbic acid nutriture assessed by serum level and subclinical symptoms in relation to daily intake. *Am. J. Clin. Nutrition*, 6: 401, 1958.
5. OKEY, R. Cholesterol content of foods. *J. Am. Dietet. A.*, 21: 341, 1945.
6. OKEY, R. Personal communication.
7. DOBBIN, E. V., GOFMAN, H. E., JONES, H. C., LYON, L. and YOUNG, C. B. The Low Fat, Low Cholesterol Diet. New York, 1953. Doubleday & Co., Inc.
8. U. S. Dept. Agriculture, Agriculture Handbook No. 8: Composition of Foods, 1950.
9. BOWES, A. DE P. and CHURCH, C. F. Food Values of Portions Commonly Used, 6th ed. Edited by Bowes, A. de P. Philadelphia, 1949.
10. BRADLEY, A. V. Tables of Food Values. Peoria, 1942. Manual Arts Press.
11. BOYD, E., EADS, M. G. and STANDSTEAD, H. R. Food Value Tables for Calculation of Diet Records. Washington, D. C., 1957. United States Public Health Service, Federal Security Agency.
12. SCHOENHEIMER, R. and SPERRY, W. M. A micromethod for the determination of free and combined cholesterol. *J. Biol. Chem.*, 106: 745, 1934.
13. WINTROBE, M. M. Clinical Hematology, 3rd ed. Philadelphia, 1951. Lea and Febiger.
14. SNEDECOR, G. W. Statistical Methods, 4th ed. Ames, 1950. The Iowa State College Press.
15. WILCOX, E. B., GILLUM, H. L. and HARD, M. M. Cooperative nutritional status studies in the western region. I. Nutrient intake. Bull. 383, Utah State Agric. College, Division of Agric. Sciences, Agricultural Experiment Station in cooperation with the Agricultural Experiment Stations of the Western States and the U. S. Dept. of Agriculture, 1956.
16. WARNICK, K. P., BRING, S. V. and WOODS, E. Nutritional status of school children 15 and 16 years of age in three Idaho communities. Research Bull. 33. Agricultural Experiment Station, College of Agriculture, University of Idaho, 1956.
17. GUILD, L. P. and ODLAND, L. M. Nutritional status of Montana fifteen-year-olds and of Montana college freshman. Bull. 536. The Human Nutrition Research Branch, U. S. Dept. of Agriculture and Montana State College, Agriculture Experiment Station, 1958.
18. WILCOX, E. B., GALLOWAY, L. S., WOOD, P. and MANGELSON, F. L. Children with and without rheumatic fever. III. Blood serum, vitamins and phosphatase data. *J. Am. Dietet. A.*, 30: 1231, 1954.
19. KRITCHENSKY, D. Cholesterol, p. 86. New York, 1958. John Wiley & Sons, Inc.
20. KORNERUP, V. Concentrations of cholesterol, total fat and phospholipid in serum of normal man. *Arch. Int. Med.*, 85: 398, 1950.
21. GILLUM, H. L., MORGAN, A. F. and JEROME, D. W. Nutritional status of the aging. IV. Serum cholesterol and diet. *J. Nutrition*, 55: 449, 1955.
22. OFFENKRANTZ, F. M. and KARSHAN, M. Serum cholesterol values for children. *Am. J. Dis. Child.*, 52: 784, 1936.
23. AFTERGOOD, L., DEUEL, H. J. JR. and ALFIN-SLATER, R. B. The comparative effects of cottonseed oil and lard on cholesterol levels in the tissues of rats. *J. Nutrition*, 62: 129, 1957.
24. PAYNE, I. R. Effect of altitude and diet on hematopoiesis and serum cholesterol. *J. Nutrition*, 64: 433, 1958.
25. SWANSON, P., LEVERTON, R., GRAM, M. R., ROBERTS, H. and PESEK, I. Blood values of women: cholesterol. *J. Gerontol.*, 10: 41, 1955.
26. SUNDERMAN, F. W. and BOERNER, F. Normal Values in Clinical Medicine. Philadelphia, 1950. W. B. Saunders Co.
27. BYERS, S. O., FRIEDMAN, M. and ROSENMAN,

R. H. Review: on the regulation of blood cholesterol. *Metabolism*, 1: 479, 1952.

28. PEELER, A. L., HEPLER, O. E., KINNEY, V. M., CISLER, L. E. and FREDERIC, T. Normal values for serum cholesterol and basal metabolic rates and their correlation in normal man. *J. Appl. Physiol.*, 3: 197, 1950.
29. FABER, H. K. and JAMES, C. A. The range of distribution of blood pressures in normal children. *Am. J. Dis. Child.*, 22: 7, 1921.
30. WILCOX, E. B., MANGELSON, F. L., GALLOWAY, L. S. and WOOD, P. Children with and without rheumatic fever. IV. Hemoglobin, packed red cells, red and white cell count, sedimentation rate, blood glucose, serum iron and copper. *J. Am. Dietet. A.*, 31: 45, 1955.

Ascorbic Acid Supplementation

I. Response of Gum Tissue

HAROLD B. PIERCE, PH.D.,* CHESTER A. NEWHALL, M.D.,† SUSAN B. MERROW, M.ED.,‡
MERTON P. LAMDEN, PH.D.,§ CRISTINA SCHWEIKER, M.S.¶ AND ALICE LAUGHLIN, M.S.‖

CLINICAL manifestations of ascorbic acid deficiency in man have been described and discussed by a number of authors.[1–12] It is generally recognized that marked changes occur in connective tissue, including gums, when there is prolonged deprivation of ascorbic acid. Many workers in the field believe that an adequate intake of ascorbic acid is necessary for the maintenance of healthy gums in man.[13–17] Vilter[11] has pointed out, however, that in experimental ascorbic acid depletion, lesions of the gum did not occur, occurred rarely or, in more prolonged studies, occurred only after tissue stores of ascorbic acid were exhausted and other scorbutic lesions had been present for some time.

Darby[10] has cautioned that gingivitis alone is not a valid criterion of ascorbic acid deficiency in the population. He further states that the gums, like other oral structures, are subject to many varieties of trauma and that numerous gingival diseases are recognized by dentists and other oral diagnosticians.

There continues to be disagreement not only regarding the specificity of gingival signs of avitaminosis C, but also on the response of physical signs associated with ascorbic acid deficiency to administered doses of this vitamin.[3,18–25] This is not surprising, because tests to assess exact degrees of deficiency are not available,[10] particularly for use with persons and groups not suffering from gross malnutrition, and also because gum tissue is continuously exposed to a wide variety of conditioning factors that are not readily controlled. Another reason for disagreement may be the diversity of opinion about the length of time necessary to effect a change in lesions of the tissue. Both Tompkins[21] and Kruse[3,25] have called attention to the limited rate at which the cumulative chronic pathology of a deficiency can be made to disappear and state it takes a long time at high nutrient levels to do so.

In a recent study[26] using sixteen correlative tests, no association was found between acute and chronic gingival lesions and serum ascorbic acid or ascorbic acid intake except that between chronic gingival lesions and serum ascorbic acid in one group of girls.

To provide information on the specificity of signs of ascorbic acid deficiency, a three-year investigation was made of changes in specific abnormalities of gum tissue in relation to each other, to ascorbic acid intake, and to serum and white cell levels of this vitamin.

Too short a period of observation and too little supplementation have been given as reasons for lack of favorable response of signs of ascorbic acid deficiency. It was believed

From the College of Medicine and the Vermont Agricultural Experiment Station, University of Vermont, Burlington, Vermont.

* Professor of Biochemistry and Chairman of Department; † Professor of Anatomy and Chairman of Department; ‡ Associate Nutritionist, Vermont Agricultural Experiment Station; § Associate Professor of Biochemistry, College of Medicine; ¶ Assistant Biochemist, Vermont Agricultural Experiment Station; ‖ Assistant Biochemist, Vermont Agricultural Experiment Station. Present address: Columbia-Presbyterian Medical Center, New York, New York.

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that, under the conditions of this study, a period of two and a half years with average daily ingestion of approximately 380 mg. of ascorbic acid per subject should be adequate for a reversal of the signs, if they were specific for ascorbic acid deficiency.

EXPERIMENTAL PROCEDURE

Subjects

The subjects were normal healthy girls, aged seventeen to twenty years, who were first year students of nursing during the fall of 1952. At this time all subjects exhibited more than one of the abnormalities of gum tissue under study. To eliminate possible extreme conditioning factors, data for subjects later showing abnormal states, such as chronic infections, various metabolic disorders or atypical dental conditions, were excluded.

In the fall of 1952, spring and fall of 1953 and 1954, and spring of 1955, photographic color transparencies were taken of the gums. In each period blood samples taken on three different days were analyzed for serum and white cell ascorbic acid, and seventeen-day diet records were secured and calculated for average daily intake of ascorbic acid, vitamin A, carotene and protein. Following the initial collection of data in the fall of 1952, the subjects were divided into two groups having comparable abnormalities of gum tissue. The test group of twenty-one subjects received ascorbic acid supplementation for two and a half years while the twenty-eight subjects in the control group were given placebos.

Diet Records

In the fall and spring of each year a record of food intake of seventeen consecutive days[27] was secured from each subject. In the majority of cases, the seventeen days fell within the period when the three blood samples were collected.

The average daily intakes of ascorbic acid, vitamin A, carotene and protein were computed using standard tables of nutrient values.[28] In attempting to obtain an estimate of the dietary distribution of vitamin A and its precursors, the vitamin A from animal foods was arbitrarily listed as vitamin A while that from plant foods was listed as carotene.

Supplementation

Each subject in the test group was instructed to take four 100 mg. ascorbic acid tablets per day. Control subjects were given placebos. A systematic program was followed for noting failures in taking ascorbic acid and assessing the actual intake.

Averages of ascorbic acid intake per day from the supplement alone were: spring 1953, 347 mg.; fall 1953, 308 mg.; spring 1954, 313 mg.; fall 1954, 331 mg.; spring 1955, 273 mg. No subject in the test group received less than an average total daily intake of 200 mg. ascorbic acid from both food and supplement in any one season.

Photography

Color transparencies of the gums were secured in the spring and fall of these three years. By using lip retractors, it was possible to photograph upper and lower gums on one transparency. The zones studied extended from canine tooth to canine tooth, inclusive. Every precaution was taken to insure consistent photographic technic in order to compare transparencies taken over a long period of time.[8]

At the time the pictures were taken, the physician associated with the study made a brief physical examination of the gums and recorded any pertinent abnormalities. Since superimposed swelling made it somewhat difficult to evaluate thickening from the picture on the screen, the physician tested for these conditions. These notations were reviewed when the transparencies were studied.

Reading of Transparencies

Data were recorded independently by the same two observers throughout the study. On comparison, any differences were given further study until the judgments of the two experienced examiners were in agreement.

Using a Kodaslide projector and the criteria[29] in Figure 1, the greatest degree of severity of the eight physical signs, regardless of the extent of the zone involved, was recorded for each of the four or six zones examined. These zone

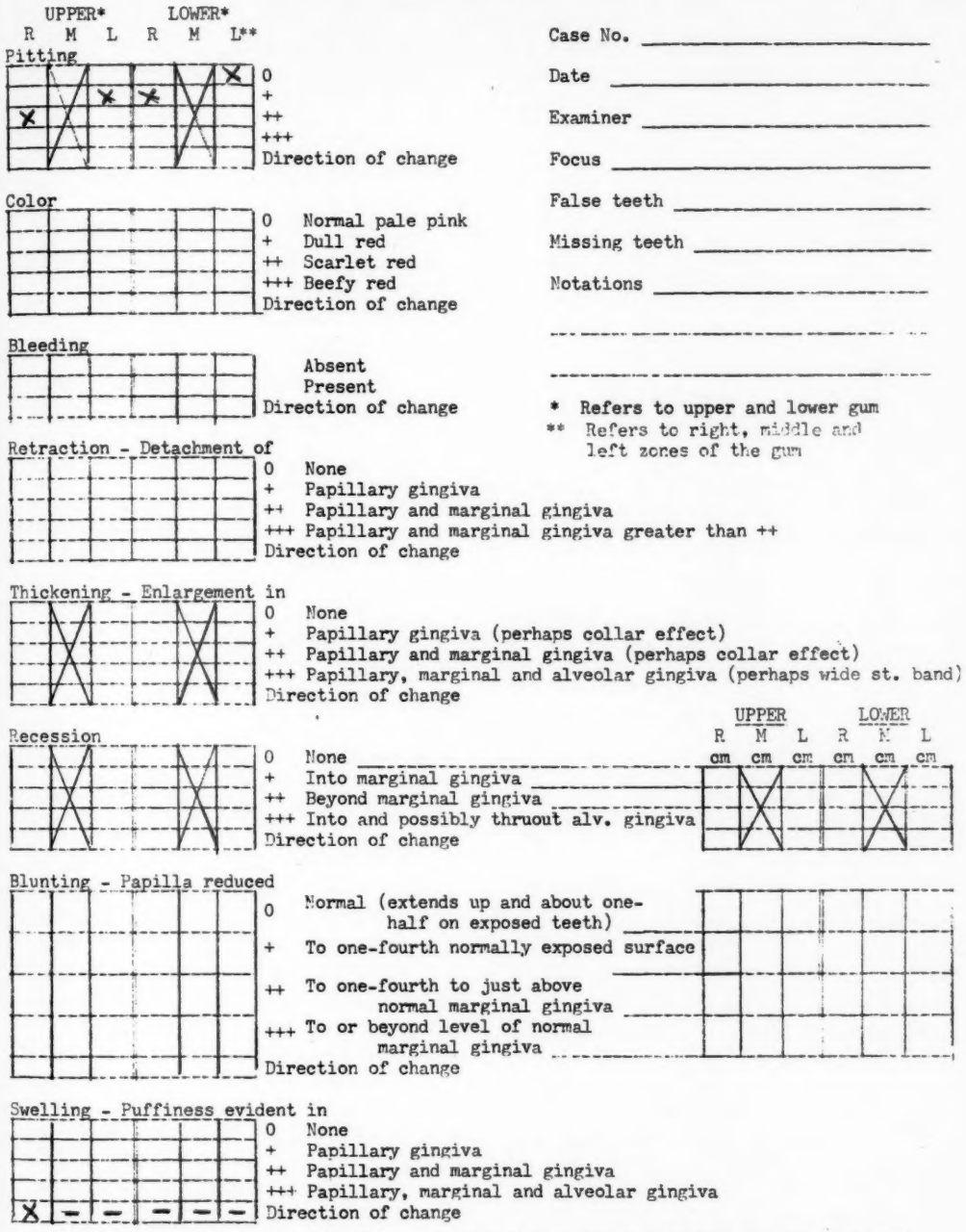

FIG. 1. Form used to record rating of transparencies of gums from midline and including canine teeth.

ratings were added to obtain the total degrees of severity for each physical sign for each subject in each of the six seasons. For example, in Figure 1, under pitting, one ++ (two plus) and two + (one plus) make a total of four degrees of severity. Values obtained for a given sign were averaged for group and seasonal comparisons. Differences in the over-all

TABLE I
Average Daily Nutrient Intake

Nutrient	Group Differences					
	F, 52	S, 53	F, 53	S, 54	F, 54	S, 55
Ascorbic acid (mg.)						
Test*	63	412	363	386	390	340
Control†	60	57	50	58	47	59
t	0.541	24.019‡	23.066‡	23.496‡	22.374‡	15.769‡
Carotene (I.U.)						
Test	3,657	3,279	2,826	2,365	2,796	2,094
Control	3,135	3,425	2,267	2,181	2,440	2,416
t	1.269	0.333	3.882‡	0.502	0.765	0.702
Vitamin A (I.U.)						
Test	3,600	3,343	3,432	3,043	3,618	2,146
Control	3,007	3,304	2,881	2,014	2,605	2,280
t	1.122	0.074	0.895	2.470§	1.744	0.297
Protein (gm.)						
Test	84.7	74.3	70.5	70.6	66.5	63.5
Control	75.2	70.9	66.0	64.2	60.4	60.0
t	2,225§	0.294	1.018	1.331	1.393	0.904

Group	Seasonal Changes			
	F, 52 to F, 54		F, 52 to S, 55	
	Difference of the Mean	t	Difference of the Mean	t
Test				
Ascorbic acid (mg.)	327	18.686‡	277	19.800‡
Carotene (I.U.)	−861	1.702	−1563	3.618‡
Vitamin A (I.U.)	18	0.026	−1454	2.808‡
Protein (gm.)	−18	3.864‡	−21	4.953‡
Control				
Ascorbic acid (mg.)	−13	2.476§	−1	0.154
Carotene (I.U.)	−695	1.821	−719	1.697
Vitamin A (I.U.)	−402	0.908	−727	1.582
Protein (gm.)	−15	3.747‡	−15	3.969‡

NOTE: In this and other tables, F = fall; S = spring.
* Twenty-one subjects.
† Twenty-eight subjects.
‡ Significant at 1% level.
§ Significant at 5% level.

tint occurred from one period to another, therefore, color changes had to be judged by comparing color differences within each individual slide. The extent of recession in each zone was determined by measuring directly on the screen the longest distance in millimeters from the tip of a tooth to the marginal tissue of the gum, and blunting was determined by measuring the length of interdental papillae.

The previously described total degrees of severity of each sign for each subject were also used to determine the amount of change which took place from fall 1952 to fall 1954, and from fall 1952 to spring 1955. The number of degrees each physical sign had increased or decreased in intensity was recorded and an average was obtained for each group.

Comparative Examination of Transparencies

At the conclusion of the investigation in

TABLE II
Averages of Differences in Degrees of Severity of Physical Signs

Season	Pitting		Color		Recession		Blunting		Swelling	
	Difference (degrees)	t	Difference (degrees)	t	Difference (degrees)	t	Difference (degrees)	t	Difference (degrees)	t
Test (21)										
F, 52 to F, 54	0.6	1.334	0.3	0.531	0.1	0.329	0.4	1.396	−0.6	0.952
F, 52 to S, 55	0.7	1.696	0.6	1.017	0.2	0.100	0.2	0.696	−1.1	1.952
Control (28)										
F, 52 to F, 54	0.3	0.983	0.4	1.081	0.1	1.354	0.6	2.745*	−0.1	0.207
F, 52 to S, 55	0.5	1.657	0.7	1.725	0.2	1.682	0.5	2.749*	−1.6	2.898†
Test vs. control										
F, 52 to F, 54	0.3	0.570	−0.1	0.219	0	0	−0.2	0.565	−0.5	0.587
F, 52 to S, 55	0.2	0.513	−0.1	0.291	0	0	−0.3	1.058	0.5	0.673

* Significant at 5% level.
† Significant at 1% level.

1955, physical signs in each subject as seen at the beginning of this study, fall 1952, were compared with corresponding physical signs, first as seen in the fall of 1954, and second, as seen in spring 1955.

For each physical sign, the four or six zones of the gum under study were examined for change, that is: a decrease (−), no change (0) or an increase (+) in the intensity and/or area involved. For each subject, and for each sign the net change was determined. The net number of zones changed −, 0 or +, was recorded for each subject and the average for each group determined. At the same time, the percentage of subjects showing a net decrease, no change or an increase was computed. The corresponding percentages for the two groups were compared to find out if they were significantly different.

Blood Chemistry

Samples of blood were obtained from the finger tips of the subjects prior to the morning meal on three different days each fall and spring within a period of two to four weeks between the first and third samples. Duplicate determinations of serum and white cell ascorbic acid[30,31] were made and the results of the three duplicate analyses for each subject in each season were averaged.

RESULTS

Changes in nutrient intake, physical signs and blood levels from fall 1952 to spring 1955, and fall 1954 are reported. Data of fall 1952 and fall 1954 were compared to avoid possible seasonal effects, although the study continued until spring 1955.

Nutrient Intake

In general, the average daily intakes of ascorbic acid, carotene, vitamin A and protein from food, reported in Table I, were similar for the two groups in the six seasons and decreased from the beginning to the end of the study. While the average intake of ascorbic acid from food did not meet the recommended allowances of the National Research Council,[32] average daily intakes of these four nutrients for both groups in all seasons were above the minimum requirements.[33]

Gum Tissue

Degrees of Severity: Since bleeding and retraction were observed in only a few subjects, these signs could not be subjected to statistical analysis.

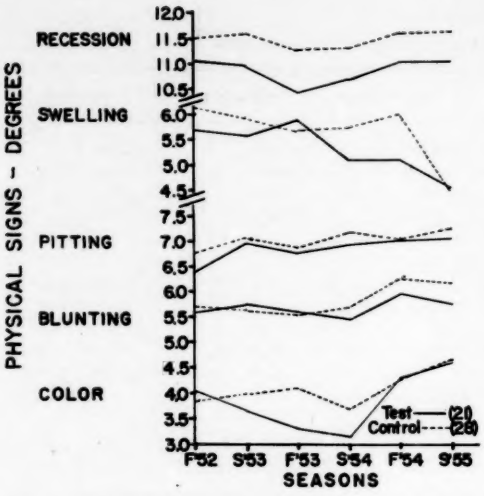

FIG. 2. Average degrees of severity of physical signs.

Thickening was prevalent in both test and control subjects. The majority had a rating of 3 degrees in each gum zone or a total of 12 degrees of severity. Only four subjects in the test group and five subjects in the control group had less than a 12-degree rating in any one of the six seasons, with no apparent difference between the two groups. It should be mentioned that appraisal of thickening was occasionally difficult because of superimposed swelling.

Orban[34] considered pitting (stippling) as a normal condition and its disappearance characteristic of progressing gingivitis. Our findings indicated that an increase in pitting might be considered an improvement, while an increase in the other signs studied was considered as indicative of further deterioration.

The average degrees of severity of physical signs in each of the two groups are shown in Figure 2. There was considerable incidence of pitting, abnormal color, recession, blunting and swelling in both test and control subjects. Comparison of the average degrees of severity of the two groups in each of the six seasons revealed no significant differences except for recession in the spring of 1953.

The average changes in degrees of severity of physical signs from the initial to each of the last two periods are presented in Table II. Pitting, color and recession did not change significantly in either the test or control group. In the control group there was a significant increase in blunting from fall 1952 to fall 1954, and from fall 1952 to spring 1955. However, when these changes were compared with the changes in the test group for the same periods, the difference between the groups in each case was not significant. The decrease in swelling in the control group from fall 1952 to spring 1955, although significant, was not different from that of the test group. As mentioned earlier, assuming that increase in pitting is indicative of improvement in condition of tissue, this sign showed a tendency of a positive effect of the supplement, since the increase in pitting was greater in the test than in the control group.

In order to determine whether a change in one physical sign was related to a change in another, correlations between the degrees of changes in physical signs were computed by the rank order correlation[35] (Table III). A positive correlation indicates that as one sign increased or decreased in degree there was a corresponding direction of change in the other signs. A negative correlation indicates an inverse relationship in the direction of change of the two signs.

Of the forty correlations, there were eleven significant positive and one significant negative correlation. Significant positive correlations occurred three times between color and recession, and between swelling and recession. The consistently positive correlations between recession and blunting, and between color and swelling are worth noting, as is the consistent negative correlation between pitting and swelling. There was also a tendency for pitting to be negatively correlated with the other signs (ten of the sixteen r_s values) and for the physical signs other than pitting, to be positively correlated with each other (twenty-three of twenty-four r_s values).

Comparing correlations of the two groups with each other, it is seen that in the test group pitting was insignificantly and negatively correlated with the other physical signs in five of eight instances. Positive correlations between other physical signs were found in eleven of

TABLE III

Rank Order Correlations of Differences in Degrees of Severity of Physical Signs and Differences in Serum and White Cell Ascorbic Acid*

Group	Color	Recession	Blunting	Swelling	Ascorbic Acid	
					Serum	White Cell
Fall 1952 to fall 1954						
Test (21)						
Pitting	+0.261	+0.211	−0.091	−0.160	−0.411	+0.094
Color	...	+0.266	−0.193	+0.258	−0.232	−0.062
Recession	...	...	+0.206	+0.203	+0.111	+0.202
Blunting	...	...	...	+0.196	+0.152	+0.110
Swelling	...	...	...	...	−0.092	−0.340
Control (28)						
Pitting	−0.158	+0.340	−0.074	−0.463	−0.162	−0.056
Color	...	+0.562†	+0.132	+0.333	+0.151	−0.026
Recession	...	...	+0.436‡	+0.436‡	+0.457‡	+0.244
Blunting	...	...	...	+0.444‡	+0.207	+0.229
Swelling	...	...	...	...	+0.167	+0.112
Fall 1952 to spring 1955						
Test (21)						
Pitting	+0.166	−0.058	−0.008	−0.031	−0.097	−0.057
Color	...	+0.476‡	+0.012	+0.444‡	+0.032	+0.254
Recession	...	...	+0.374	+0.480‡	+0.175	+0.255
Blunting	...	...	...	+0.442‡	+0.156	+0.068
Swelling	...	...	...	...	+0.028	+0.127
Control (28)						
Pitting	−0.158	+0.250	+0.156	−0.321	−0.159	0.000
Color	...	+0.508†	+0.041	+0.566†	+0.045	−0.217
Recession	...	...	+0.370	+0.614†	+0.346	+0.344
Blunting	...	...	...	+0.242	0.000	+0.253
Swelling	...	...	...	...	−0.074	+0.075

* r_s values.
† Significant at 1% level.
‡ Significant at 5% level.

twelve instances, four being significant. In the control group, pitting was negatively correlated with other physical signs in five of eight instances, one being significant. Positive correlations between the other physical signs were found in all of twelve instances, seven of these being significant. These data indicate that there was relatively little difference in the pattern of correlations between the two groups.

Direction of Change of Physical Signs: As previously described, a study was made of the direction of change from the initial to each of the last two periods. That is, the examiners determined whether there had been a decrease (−), no change (0) or an increase (+), in the intensity and/or area involved. Data on thickening have been included because changes were apparent when this sign was rated in this manner. There was no significant difference between the two groups in the average net change in zones for each physical sign. As in the previous comparison, therapy appeared to have no significant effect on individual physical signs when the data were analyzed statistically. Although the study was primarily of individual physical abnormalities of the gum tissue it is interesting to note that when the data for the average net physical changes of the test group from fall 1952 to fall 1954 were pooled, providing an over-all evaluation characteristic of

the gingivitis syndrome, there was a significant difference between the test and the control groups. No significant difference was found in the pooled comparison of average net changes in individual signs from fall 1952 to spring 1955.

When the percentages of subjects with net change of −, 0 or +, in the test group were compared with the percentages in the control group by the chi-square test,[36] a significant difference was found for only one sign, recession, fall 1952 to fall 1954. The pooled data showed the difference between groups to be significant on net changes from fall 1952 to fall 1954. They were not significant from fall 1952 to spring 1955.

The findings on correlations between net changes in physical signs by the rank order correlation revealed that of the sixty correlations forty were positive, nine significantly so, and twenty were negative, three significantly so. Four of these nine significant positive correlations were between color and swelling while three significant negative correlations were between pitting and swelling. There is a definite tendency toward a negative correlation between pitting and the other five physical signs (sixteen of the twenty r_s values) and of a positive correlation between all physical signs except pitting (thirty-six of forty r_s values).

Blood Levels and Physical Findings

This section is concerned largely with correlations between ascorbic acid blood levels and physical findings. Treatment of these levels with respect to other factors such as intake, season and other nutrients will be found in the second part of this report.[37]

That the daily ascorbic acid supplement was ingested and absorbed by the test group, making it different from the control group and presumably in a better position to improve any condition of the gums conceivably related to insufficiency of ascorbic acid, is indicated by the significantly increased levels of serum and white cell ascorbic acid. This is in agreement with the commonly accepted finding that serum ascorbic acid levels reflect ascorbic acid intake.

There was only one significant correlation between changes in serum and white cell ascorbic acid and changes in physical signs. This was between a change in the serum ascorbic acid and degree of change in the physical sign, recession, from fall 1952 to fall 1954 in the control group (Table III). Considering all the correlations, those between pitting and serum ascorbic acid were all negative while those between the other physical signs and serum ascorbic acid tended to be positive (twelve of the sixteen r_s values). Two of four correlations between pitting and white cell ascorbic acid were negative with a tendency to a positive correlation between the other physical signs and white cell ascorbic acid (twelve of sixteen r_s values). All four correlations between changes in serum and white cell ascorbic acid were positive. The positive correlation in the test group from fall 1952 to spring 1955 was significant and approached significance from fall 1952 to fall 1954.

The net change in color from fall 1952 to fall 1954 in the control group showed a significant positive correlation with serum ascorbic acid but a significant negative correlation between color and white cell ascorbic acid in the test group. There were two negative correlations between net changes in pitting and changes in serum ascorbic acid, and no negative correlation between net changes in pitting and changes in white cell ascorbic acid. For the twenty correlations between the other physical signs and serum, ten were positive. There were eleven positive correlations between physical sign changes and changes in white cell ascorbic acid.

SUMMARY AND CONCLUSIONS

To provide information on the question of the specificity of signs of ascorbic acid deficiency, a three-year investigation was made of changes in gum tissue (pitting, color, bleeding, retraction, thickening, recession, blunting, swelling) in relation to ascorbic acid intake and white cell and serum levels of this vitamin. Forty-nine female student nurses showing abnormalities of gum tissue were divided into two groups. The control group, which received placebos, had an average daily intake of ascorbic acid from 47 to 60 mg., and the test group, with an ascorbic acid supplement, had a

total average daily intake of ascorbic acid from 340 to 412 mg. The gum tissue was observed over a period of two and a half years to determine whether one or more of the signs would respond to the supplement and whether these signs were correlated with each other and/or serum and white cell levels of this vitamin.

The following statements summarize the major findings:

Under the conditions of this study, the signs studied (pitting, color, thickening, recession, blunting, swelling) are not specific for ascorbic acid deficiency.

Incidence of bleeding and retraction was negligible. Thickening was prevalent and did not change with supplementation over the two and a half-year period.

Although there was considerable incidence of pitting, swelling, blunting, recession and abnormal color in both the test and control groups there was no significant effect of supplement on the average degrees of severity of these five signs nor were the changes from the initial to final periods significantly different between the test and control groups.

There was a definite tendency toward a negative correlation between pitting and the other five physical signs and of a positive correlation between all physical signs except pitting unrelated to the ascorbic acid supplement. There were few significant correlations between changes in serum and white cell ascorbic acid and changes in physical signs.

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REFERENCES

1. Crandon, J. H., Lund, C. C. and Dill, D. B. Experimental human scurvy. *New England J. Med.*, 223: 353, 1940.
2. Ralli, E. P. and Sherry, S. Adult scurvy and metabolism of vitamin C. *Medicine*, 20: 251, 1941.
3. Kruse, H. D. Gingival manifestations of avitaminosis C, with especial consideration of the detection of early changes by biomicroscopy. *Milbank Mem. Fund Quart.*, 20: 290, 1942.
4. Pijoan, M. and Lozner, E. L. Medical progress; physiologic significance of vitamin C in man. *New England J. Med.*, 231: 14, 1944.
5. King, J. D. Gingival disease in Dundee. *Dent. Rec.*, 65: 9, 32, 55, 1945.
6. Leeson, H. J., Webb, J. F., Ferguson, H. P., Semmons, E. M. and McHenry, E. W. Study of ascorbic acid nutrition. *Canad. M. A. J.*, 53: 341, 1945.
7. Council on Foods and Nutrition. Vitamin deficiencies: stigmas, symptoms, and therapy. *J. A. M. A.*, 131: 666, 1946.
8. Linghorne, W. J., McIntosh, W. G., Tice, J. W., Tisdall, F. F., McCreary, J. F., Drake, T. G. H., Greaves, A. V. and Johnstone, W. M. Relation of ascorbic acid intake to gingivitis. *Canad. M. A. J.*, 54: 106, 1946.
9. Medical Research Council. Vitamin C requirement of human adults. Experimental study of vitamin C deprivation in man. A preliminary report by the vitamin C subcommittee of the Accessory Food Factors Committee. *Lancet*, 1: 853, 1948.
10. Darby, W. J. Evaluation of symptoms and signs of deficiency diseases. *Fed. Proc.*, 9: 592, 1950.
11. Vilter, R. W. Effects of deficiency in human beings. In: The Vitamins. I, p. 348. New York, 1954. Academic Press, Inc.
12. Ogilvie, G. F. and Pett, L. B. Long term study on ascorbic acid supplementation. *Canad. Serv. M. J.*, 10: 191, 1954.
13. Jolliffe, N., Goodhart, R. S., Archer, M., Lopez, H. and Dias, F. G. Nutrition status survey of the sixth grade school population of Cuba. *J. Nutrition*, 64: 355, 1958.
14. Abt, A. F. and Farmer, C. J. Vitamin C. In: Pharmacology and Therapeutics in the Vitamins, p. 431. Chicago, 1939. American Medical Association.
15. Jolliffe, N., Tisdall, F. F. and Cannon, P. R. Clinical Nutrition, p. 68. New York, 1950. Paul B. Hoeber, Inc.
16. McLester, J. S. and Darby, W. J. Scurvy (vitamin C deficiency). In: Nutrition and Diet in Health and Disease, p. 266. Philadelphia, 1952. W. B. Saunders Co.
17. Shaw, J. H. Nutrition in relation to dental medicine. In: Modern Nutrition in Health and Disease, p. 520. Philadelphia, 1955. Lea & Febiger.
18. Burrill, D. Y. Relationship of blood plasma vitamin C level to gingival and peridontal disease. *J. Dent. Res.*, 21: 353, 1942.

19. STAMM, W. P., MACRAE, T. F. and YUDKIN, S. Incidence of bleeding gums among R. A. F. personnel and the value of ascorbic acid in treatment. *Brit. M. J.*, 2: 239, 1944.
20. BRANSBY, E. R., BURN, J. L., MAGEE, H. E. and MACKECKNIE, D. M. Effect of a daily vitamin supplement on the health and development of children. *Brit. M. J.*, 1: 193, 1946.
21. TOMPKINS, W. T. Objective evidences of nutritional deficiencies in pregnancy. In: Nutrition in Relation to Health and Disease, p. 82. New York, 1950. Milbank Memorial Fund.
22. OCKERSE, T., LERICHE, H., KINNEAR, A. A. and DRAPER, W. L. Nutrition and health study on Bantu boys. *New York Dent. J.*, 19: 213, 1953.
23. MORRIS, G. E. Avitaminosis C in 1953: clinical study. *Am. Pract. & Digest Treat.*, 5: 658, 1954.
24. Gingivitis and glossitis of undetermined origin. In: Special Issue on Nutrition and Disease. Edited by Spies, T. D. *Postgrad. Med.*, 17: 27, 1955.
25. KRUSE, H. D. Concept of etiological complex of deficiency states with especial consideration of conditions. *Milbank Mem. Fund Quart.*, 27: 5, 1949.
26. HARD, M. M., ESSELBAUGH, N. C. and DONALD, E. A. Nutritional status of selected adolescent children. III. Ascorbic acid nutriture assessed by serum level and subclinical symptoms in relation to daily intake. *Am. J. Clin. Nutrition*, 6: 401, 1958.
27. YOUNG, C. M., CHALMERS, F. W., CHURCH, H. M., CLAYTON, M. M., GATES, L. O., HAGAN, G. C., STEELE, B. F., TUCKER, R. E., WERTZ, A. W. and FOSTER, W. D. Northeast Regional Publication No. 10. III. Dietary Methodology Studies. *Massachusetts Agr. Exper. Sta. Bull. No. 469*, 1952, p. 39.
28. WATT, B. K. and MERRILL, A. L. Composition of foods—raw, processed, prepared. U. S. Dept. of Agriculture, Agricultural Handbook, No. 8. Washington, D. C., 1950.
29. NEWHALL, C. A., MERROW, S. B., DURBROW, A. B., LAMDEN, M. P. and PIERCE, H. B. On physical stigmata ascribed to vitamin A and ascorbic acid deficiencies. *Am. J. Clin. Nutrition*, 1: 517, 1953.
30. LOWRY, O. H., LOPEZ, J. A. and BESSEY, O. A. Determination of ascorbic acid in small amounts of blood serum. *J. Biol. Chem.*, 160: 609, 1945.
31. BESSEY, O. A., LOWRY, O. H. and BROCK, M. J. Quantitative determination of ascorbic acid in small amounts of white blood cells and platelets. *J. Biol. Chem.*, 168: 197, 1947.
32. Food and Nutrition Board. Recommended Dietary Allowances. Nat. Acad. Sc.—Nat. Res. Council, Publ. 589. Washington, D. C., revised 1958.
33. Federal Register: Federal Food Drug and Cosmetic Act. Section 403(j), November, 1941.
34. ORBAN, B. Clinical and histologic study of the surface characteristics of the gingiva. *Oral Surg.*, 1: 827, 1948.
35. SNEDECOR, G. A. Statistical Methods, 4th ed., p. 164. Ames, Iowa, 1946. Iowa State College Press.
36. SNEDECOR, G. W. Statistical Methods, 4th ed., p. 192. Ames, Iowa, 1946. Iowa State College Press.
37. LAMDEN, M. P., MERROW, S. B., PIERCE, H. B., SCHWEIKER, C., NEWHALL, C. A. and LAUGHLIN, A. Ascorbic acid supplementation. II. Response of certain blood constituents. *Am. J. Clin. Nutrition*, 8: 363, 1960.

Ascorbic Acid Supplementation

II. Response of Certain Blood Constituents

MERTON P. LAMDEN, PH.D.,* SUSAN B. MERROW, M.ED.,† HAROLD B. PIERCE, PH.D.,‡
CRISTINA SCHWEIKER, M.S.,§ CHESTER A. NEWHALL, M.D.¶ AND ALICE LAUGHLIN, M.S.||

THE EFFECT of ascorbic acid intake from foods and supplements on serum and white cell levels of this vitamin has been reported by numerous investigators.[1–12] In general, where intake and blood levels have been low, increased ingestion has been followed by an elevation of the blood levels. On the other hand, when intake and blood levels have initially been relatively high, supplements as large as 1 gm. per day for three months have resulted in no progressive change in either the serum or white blood cell levels.[9]

This paper presents an analysis of the data on intake and blood levels of certain nutrients obtained during a three-year study of the response of lesions of the gums to ascorbic acid supplementation.[13] Serum levels of ascorbic acid, vitamin A, carotene and protein, and white cell ascorbic acid levels were studied in relation to the intakes of these same nutrients.

EXPERIMENTAL PROCEDURE

Duplicate determinations were made of serum and white cell ascorbic acid,[14,15] serum vitamin A, carotene[16] and protein,[17] and ar average was obtained of the analyses of three blood samples for each season. Details of this investigation, such as blood collection, selection of subjects and estimation of nutrient intake have been described in the preceding paper.[13]

A study of the urinary excretion of ascorbic acid was conducted with nine subjects. In the fall of 1952, subjects having high, medium and low serum ascorbic acid levels, according to the initial blood determinations, were selected from the prospective test group. The twenty-four–hour excretion of ascorbic acid was determined by a modification of the 2,4-dinitrophenylhydrazine method[18] one week before, on the first day, and after two, five and twenty-seven months of supplementation. Except for the first day of supplementation, values are averages of two successive twenty-four–hour urine collections. Serum and white cell ascorbic acid levels were also determined on the days urine samples were collected.

RESULTS

The data collected in the initial period, fall 1952, were used as a baseline for comparisons of changes that occurred in the five subsequent periods through spring 1955. Although interest was primarily in changes from the beginning to the end of the study, comparisons were also made from fall 1952 to fall 1954 to allow for a possible seasonal effect.

Ascorbic Acid Intake

The average daily intake of ascorbic acid from food (Table I) was approximately the same for test and control groups at the begin-

From the College of Medicine and the Vermont Agricultural Experiment Station, University of Vermont, Burlington, Vermont.

* Associate Professor of Biochemistry, College of Medicine; † Associate Nutritionist, Vermont Agricultural Experimental Station; ‡ Professor of Biochemistry and Chairman of Department; § Assistant Biochemist, Vermont Agricultural Experiment Station; ¶ Professor of Anatomy and Chairman of Department; || Assistant Biochemist, Vermont Agricultural Experiment Station. Present address: Columbia-Presbyterian Medical Center, New York, New York.

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TABLE I
Average Ascorbic Acid Intake

Season and Year	Test Group (mg.)	Control Group (mg.)	Significance of Difference Between Groups (%)
F, 52	63 ± 3*	60 ± 4*	n.s.
S, 53	412 ± 17	57 ± 3	1
F, 53	363 ± 15	50 ± 4	1
S, 54	386 ± 17	58 ± 4	1
F, 54	390 ± 17	47 ± 3	1
S, 55	340 ± 20	59 ± 5	1

NOTE: In this and other tables, F = fall; S = spring.
* Standard errors.

ning of the study. During supplementation the total average ascorbic acid intake per day of the test group was six to eight times greater than that of the control group.

Ascorbic Acid Content of Serum and White Blood Cells

Tables II and III show the average ascorbic acid contents of the serum and white cells of the test and control groups, and comparisons between the two groups in each of the six seasons. In the fall of 1952, before the supplement was started, the average values of serum and white cell ascorbic acid were in the middle of the range of average levels reported for adults.[12,19–26] At this time the average serum ascorbic acid level for the test group was significantly higher ($P < 0.05$) than that of the control group. In each of the subsequent periods, the average serum ascorbic acid levels of the test group were significantly higher ($P < 0.01$) than those of the control group and the differences between the two groups were considerably greater than in the initial period. In the fall of 1952 the average white cell ascorbic acid levels of the two groups were approximately the same, but with supplementation these levels were significantly higher for the test than for the control group in all periods except the fall of 1953.

TABLE II
Average Serum Ascorbic Acid Levels

Season and Year	Test Group (mg. %)	Control Group (mg. %)	Significance of Difference Between Groups (%)
F, 52	1.17 ± 0.07*	0.98 ± 0.05*	5
S, 53	2.03 ± 0.10	1.26 ± 0.07	1
F, 53	1.87 ± 0.11	1.29 ± 0.06	1
S, 54	2.04 ± 0.07	1.36 ± 0.07	1
F, 54	1.57 ± 0.12	1.08 ± 0.06	1
S, 55	1.58 ± 0.12	1.17 ± 0.06	1

* Standard errors.

TABLE III
Average White Blood Cell Ascorbic Acid Levels

Season and Year	Test Group (mg. %)	Control Group (mg. %)	Significance of Difference Between Groups (%)
F, 52	28.67 ± 1.12*	28.39 ± 0.71*	n.s.
S, 53	31.67 ± 1.10	28.21 ± 0.89	5
F, 53	32.95 ± 1.26	30.07 ± 1.16	n.s.
S, 54	32.40 ± 1.59	25.90 ± 1.49	1
F, 54	32.38 ± 2.52	25.32 ± 1.41	5
S, 55	36.52 ± 1.71	28.07 ± 1.08	1

* Standard errors.

The average serum levels of the test group were significantly higher in the fall of 1954 ($P < 0.01$) and the spring of 1955 ($P < 0.01$) than in the fall of 1952. The average serum level of the control group was also significantly higher ($P < 0.05$) in the spring of 1955 than in the fall of 1952 but this cannot be attributed to an increased intake of ascorbic acid.

The average white cell ascorbic acid level of the test group was higher in the two final periods than in the fall of 1952. The change from fall 1952 to spring 1955 was significant ($P < 0.01$). In the control group there was less, but not significantly less, ascorbic acid in the white cells in the final periods than in the base period.

It is apparent that the supplement taken by the test group resulted in an increase in both serum and white cell ascorbic acid levels over the entire period.

Ascorbic Acid Correlations

No significant correlations were found between ascorbic acid intake and white cell ascorbic acid level. With the exception of the

control group in the fall of 1952, the positive correlations between ascorbic acid intakes and serum ascorbic acid levels were significant [r values: test group +0.436 ($P < 0.05$), +0.454 ($P < 0.05$), +0.509 ($P < 0.05$); control group +0.549 ($P < 0.01$), +0.376 ($P < 0.05$)]. Similar results were also found in nutritional status studies conducted in the northeast region[27] and by Hard et al.[28] On the other hand, Dodds and MacLeod[29] found limited association between dietary ascorbic acid and serum ascorbic acid levels. However, in their study, intakes were based on dietary records of only one or two days. Bryan et al.[1] reported a correlation between ascorbic acid intake and plasma ascorbic acid level only when this level was less than 1.0 mg. per cent.

The correlations between serum and white cell ascorbic acid levels were not significant except for the control group in the spring of 1955 ($r = +0.495$, $P < 0.01$). Morse et al.[12] found significant positive correlations when adult women subjects were receiving ascorbic acid intakes of 33 mg., 58 mg. and 83 mg. but not when they were receiving 133 mg. of ascorbic acid. In a second study, Morse et al.[30] reported no significant correlations with intakes over 47 mg. These findings suggest that significant correlations between serum and white cell levels would be unlikely at the intake levels of both the test and control groups in the present study.

Rank correlation coefficients[31] indicated there was no significant correlation between changes from the base period to the two final periods in ascorbic acid intakes and white cell levels in either group. A significant positive correlation between changes in ascorbic acid intakes and in serum ascorbic acid levels occurred only in the test group in the period from fall 1952 to spring 1955 ($r_s = +0.696$, $P < 0.01$).

There was a significant positive correlation ($r_s = +0.433$, $P < 0.05$) between changes in serum and white cell ascorbic acid levels of the test group from fall 1952 to spring 1955 and a positive correlation ($r_s = +0.405$) significant at the 10 per cent level between changes from fall 1952 to fall 1954. That is, the subjects with the greatest increase in serum ascorbic acid tended to be the subjects with the greatest increase in white cell ascorbic acid. Whereas the correlation between the serum and white cell levels in one season was not significant (with one exception), as previously noted, the rank correlation determined for *changes* in levels caused by supplementation tended to be significant.

Nutrient Interrelationships

Relations between the intakes of vitamin A, carotene, and protein and their respective levels in the serum were also investigated.

The average dietary intakes of vitamin A and carotene of both groups were essentially similar in all seasons. For the most part, the combined intake of vitamin A and carotene exceeded the recommended dietary allowance of the National Research Council. The serum carotene levels in both groups showed a consistent seasonal pattern with values for the spring lower than those of the previous fall. There was no significant difference between the serum vitamin A or carotene levels of the test and control groups in any season, and the ascorbic acid supplement seemed to have no significant effect on the average serum levels of vitamin A or carotene. Analysis of the data suggested a positive association between changes in serum vitamin A and carotene levels unrelated to the ascorbic acid supplement.

The average protein intake of each group was initially above the recommended dietary allowance of the National Research Council and decreased significantly to below this allowance during the period of the investigation. The average serum protein was the same for each group throughout the study and decreased significantly over this period, but no consistent correlation was found between protein intake and serum protein level or between changes in intake and serum level. This indicates that, although the average serum protein level of the group decreased when the average protein intake decreased, it would not be possible to predict the serum protein level from the protein intake or the extent to which a decrease in protein intake of a subject would be reflected in a decrease in the serum protein level.

In order to uncover any possible interrelations, the data were further analyzed. Rank

correlation coefficients were determined for all the remaining possible associations on changes between and among the diet and blood constituents studied. Although occasional significant correlations occurred, there seemed to be no consistent pattern.

Ascorbic Acid Excretion

Urinary excretion of ascorbic acid in the nine selected subjects averaged 34 mg., 56 mg., 384 mg., 333 mg. and 332 mg. one week before, on the first day, and after two, five, and twenty-seven months of supplementation, respectively. The increase in ascorbic acid excretion after the first day of supplementation was significant ($P < 0.01$). The decreases in urinary excretion of ascorbic acid on the last two sampling dates were not significant.

During the first day of supplementation there was only a slight rise in the urinary excretion of ascorbic acid indicating that, on the average, these subjects were unsaturated with respect to ascorbic acid. Considering the average daily ascorbic acid intake of 63 mg. as indicated by seventeen-day diet records and the serum ascorbic acid level of 1.21 mg. per cent (range 0.96 to 1.60 mg. per cent), a greater increase might have been expected. Roderuck et al.,[32] however, reported that there may be a lower correlation between mean intake and either blood concentration or urinary excretion if the daily intake is irregular. The situation in this study may have been similar. The initial average daily intake from the seventeen-day diet records ranged from 42 to 74 mg. and individual ascorbic acid intakes recorded for the days during which samples were collected varied widely from the average calculated from the seventeen-day diet records.

Within the individual sampling dates there were no significant correlations between the urinary excretion of ascorbic acid and the intake or white cell levels. When the data on urinary excretion and intake before supplementation and after two, five and twenty-seven months of supplementation were pooled, there was a significant positive correlation ($r = +0.805$, $P < 0.01$). The only significant correlation ($r = +0.855$, $P < 0.01$) between the urinary excretion and serum levels of ascorbic acid occurred on the first day of supplementation.

SUMMARY AND CONCLUSIONS

The effect of ascorbic acid supplementation on the serum and white cell ascorbic acid levels, the serum levels of vitamin A, carotene and protein, and the urinary excretion of ascorbic acid of women of college age was studied for three years. Serum and white cell ascorbic acid levels of the test group which received large daily doses of ascorbic acid were significantly higher than those of the control group. There were significantly higher levels for the test group in the final periods than in the base period. The subjects in the test group having the greatest increase in serum ascorbic acid tended to have the greatest increase in white cell ascorbic acid levels. The supplement did not affect the serum levels of vitamin A, carotene and protein. In both the test and control subjects, the ascorbic acid intakes were significantly correlated with the serum ascorbic acid levels. The amount of change in one was related to the amount of change in the other only in the test group from the base to the final period.

The ascorbic acid supplement greatly increased the urinary excretion of this nutrient throughout the study. There was a significant positive correlation between urinary excretion and fasting serum levels of ascorbic acid on the first day of supplementation.

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REFERENCES

1. Bryan, A. H., Turner, D. F., Huenemann, R. L. and Lotwin, G. Relation between plasma and dietary ascorbic acid. *Am. J. M. Sc.*, 202: 77, 1941.
2. Bessey, O. A. and White, R. L. Ascorbic acid re-

quirements of children. *J. Nutrition*, 23: 195, 1941.

3. Storvick, C. A. and Hauck, H. M. Effect of controlled ascorbic acid ingestion upon urinary excretion and plasma concentration of ascorbic acid in normal adults. *J. Nutrition*, 23: 111, 1942.
4. Fincke, M. L. and Landquist, V. L. Daily intake of ascorbic acid required to maintain adequate and optimal levels of this vitamin in blood plasma. *J. Nutrition*, 23: 483, 1942.
5. Dodds, M. L. and MacLeod, F. L. Survey of the ascorbic acid status of college students. *J. Nutrition*, 27: 315, 1944.
6. Proceedings of Conference on Methods for Evaluating Nutritional Status of Mothers, Infants and Children, p. 54. Research Laboratory of the Children's Fund of Michigan. Detroit, 1947.
7. Hollinger, M. E. and Attaya, R. B. Comparison of the ascorbic acid content of plasma and of whole blood as criteria of nutritional status. *J. Nutrition*, 37: 203, 1949.
8. Urbach, C., Hickman, K. C. D. and Harris, P. L. Effect of individual vitamins A, C, E and carotene administered at high levels on their concentration in the blood. *Exper. Med. & Surg.*, 10: 7, 1952.
9. Lowry, O. H., Bessey, O. A. and Burch, H. B. Effect of prolonged high dosage with ascorbic acid. *Proc. Soc. Exper. Biol. & Med.*, 80: 361, 1952.
10. Fisher, K. H. and Dodds, M. L. Variability in the measure of total ascorbic acid utilization by the human. *J. Nutrition*, 54: 389, 1954.
11. Potgieter, M., Morse, E. H. and Walker, G. R. Ascorbic acid utilization by women. Response of serum level and night urinary excretion to increasing levels of intake. *J. Nutrition*, 55: 217, 1955.
12. Morse, E. H., Potgieter, M. and Walker, G. R. Ascorbic acid utilization by women. Response of blood serum and white blood cells to increasing levels of intake. *J. Nutrition*, 58: 291, 1956.
13. Pierce, H. B., Newhall, C. A., Merrow, S. B., Lamden, M. P., Schweiker, C. and Laughlin, A. Ascorbic acid supplementation. I. Response of gum tissue. *Am. J. Clin. Nutrition*, 8: 353, 1960.
14. Lowry, O. H., Lopez, L. A. and Bessey, O. A. Determinations of ascorbic acid in small amounts of blood serum. *J. Biol. Chem.*, 160: 609, 1945.
15. Bessey, O. A., Lowry, O. H. and Brock, M. J. Quantitative determination of ascorbic acid in small amounts of white blood cells and platelets. *J. Biol. Chem.*, 168: 197, 1947.
16. Bessey, O. A., Lowry, O. H. and Brock, M. J. Determination of vitamin A and carotene in small quantities of blood serum. *J. Biol. Chem.*, 166: 177, 1946.
17. Lowry, O. H. and Hunter, T. H. Determination of serum protein concentration with a gradient tube. *J. Biol. Chem.*, 159: 465, 1945.
18. Roe, J. H. and Oesterling, M. J. Determination of dehydroascorbic acid and ascorbic acid in plant tissues by the 2,4-dinitrophenylhydrazine method. *J. Biol. Chem.*, 152: 511, 1944.
19. Clayton, M. M., Babcock, M. J., Foster, W. D., Stregevsky, S., Tucker, R. E., Wertz, A. W. and Williams, H. H. Cooperative nutritional status studies in the northeast region. V. Blood findings. *Bull. 516, Maine Agr. Exper. Sta.*, 1953.
20. Lowry, O. H., Bessey, O. A., Brock, M. J. and Lopez, J. A. Interrelationship of dietary, serum, white blood cell and total body ascorbic acid. *J. Biol. Chem.*, 166: 111, 1946.
21. Bodansky, O., Wroblewski, F. and Markardt, B. Concentration of ascorbic acid in plasma and white cells of patients with cancer and noncancerous chronic disease. *Cancer*, 5: 678, 1952.
22. Waldo, A. L. and Zipf, R. E. Ascorbic acid in level leukemia patients. *Cancer*, 8: 187, 1955.
23. West, E. S. and Todd, W. R. Textbook of Biochemistry, p. 791. New York, 1952. Macmillan Co.
24. Butler, A. M., Cushman, M. and MacLachlan, E. A. Determination of ascorbic acid in whole blood and its constituents by means of methylene blue; macro- and micro-methods. *J. Biol. Chem.*, 150: 453, 1943.
25. Rourke, G. M., MacLachlan, E. A. and Butler, A. M. Case records of the Massachusetts General Hospital. Normal laboratory values. Blood, plasma or serum values. *New England J. Med.*, 234: 24, 1946.
26. Lubschez, R. Studies in ascorbic acid with especial reference to the white layer. I. Description of method and comparison of ascorbic acid levels in whole blood, plasma, red cells, and white layer. *J. Clin. Invest.*, 24: 573, 1945.
27. Babcock, M. J., Clayton, M. M., Foster, W. D., Lojkin, M. E., Tucker, R. E., VanLandingham, A. H. and Young, C. M. Cooperative nutritional status studies in the northeast region. VI. Correlations. *West Virginia Agr. Exper. Sta., Bull. 361T*, 1953.
28. Hard, M. M., Esselbaugh, N. C. and Donald, E. A. Nutritional status of selected adolescent children. III. Ascorbic acid nutriture assessed by serum level and subclinical symptoms in relation to daily intake. *Am. J. Clin. Nutrition*, 6: 401, 1958.
29. Dodds, M. L. and MacLeod, F. L. Survey of the ascorbic acid status of college students. *J. Nutrition*, 27: 315, 1944.
30. Morse, E. H., Potgieter, M. and Walker, G. R. Ascorbic acid utilization by women. Response

of blood serum and white cells to increasing levels of intake in two groups of women of different age levels. *J. Nutrition*, 60: 229, 1956.

31. SNEDECOR, G. W. Statistical Methods, 4th ed., p. 164. Ames, Iowa, 1946. Iowa State College Press.

32. RODERUCK, C., BURRILL, L., CAMPBELL, L. J., BRAKKE, B. E., CHILDS, M. T., LEVERTON, R., CHALOUPKA, M., JEBE, E. H. and SWANSON, P. P. Estimated dietary intake, urinary excretion and blood vitamin C in women of different ages. *J. Nutrition*, 66: 15, 1958.

Effect of D-Glucuronic Acid and D-Glucuronolactone on Ascorbic Acid Levels in Blood and Urine of Man and Dog

EUGENE M. BAKER, PH.D., EDWIN L. BIERMAN, M.D. AND IRVIN C. PLOUGH, M.D.

IN 1953 Horowitz and King[1] demonstrated that the injection of C^{14}-labeled D-glucuronolactone in rats led to the formation of C^{14}-labeled L-ascorbic acid. Isherwood et al.[2] later showed that D-glucuronic acid was converted first to L-gulonic acid and then to L-ascorbic acid. Recently Touster et al.[3] further demonstrated that xylulose was excreted by human beings and guinea pigs fed large amounts of D-glucuronolactone.

That D-glucuronate was first converted to L-gluonate, then to 3-keto-L-gulonate and finally to L-ascorbate was demonstrated by Bublitz et al.,[4] using surviving tissue slices. In certain instances 3-keto-L-gulonate was decarboxylated and converted to L-xylulose.

During the course of studies in man on the renal excretion of ketopentoses, chiefly xylulose, it was noted that marked increases occurred in ascorbic acid levels in blood and urine following the ingestion of D-glucuronolactone. Therefore, it was decided to study the effects of the administration of D-glucuronolactone and D-glucuronic acid both in man and in a dog.

When given lactone all subjects excreted excess ascorbate, whereas when given the D-glucuronic acid only the dog excreted excess ascorbate. This suggests that man may synthesize ascorbic acid from certain precursors.

From the U. S. Army Medical Research and Nutrition Laboratory, Fitzsimmons Army Hospital, Denver, Colorado.

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MATERIAL AND METHODS

D-Glucuronolactone and D-glucuronic acid used in the study were checked for purity by melting point measurement and by paper chromatography.

The method for measuring ascorbic acid levels in plasma and urine was that of Schaffert and Kingsley.[5] Urine ketopentose (xylulose) was determined by the procedure described by Futterman and Roe[6] as modified by Baker et al.[7]

The chromatographic method, except for a few details, was that of Mapson and Partridge.[8] Whatman No. 3 sheets were used in a descending solvent system of phenol, glacial acetic acid and water (100:1:100 by volume) and run in the presence of 10 to 20 mg. NaCN for a period of twenty to twenty-two hours. Ascorbic acid in an acid medium was shown to withstand shell freezing and lyophilization to dryness without loss or destruction. Therefore, since a spot of 40 μg. or more was required for positive identification, one-tenth of each urine sample was shell frozen, lyophilized to dryness and then the residue dissolved in 1 ml. of water; 0.1 ml. was immediately applied to the chromatographic sheets. A standard of ascorbic acid was dissolved in urine, as it was suspected that urine salts could possibly alter the R_f values.

Four men and two women served as experimental subjects. They fasted from midnight

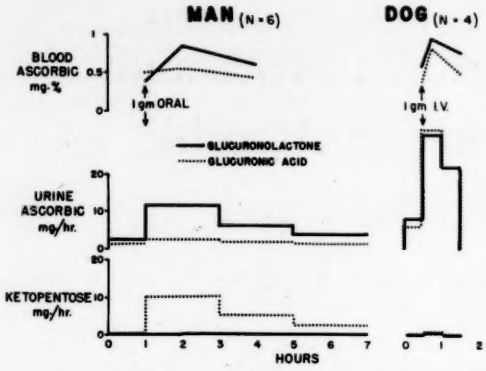

FIG. 1. Ascorbic acid levels in blood and urine, and ketopentose levels in urine, in man and dog following administration of D-glucuronolactone and D-glucuronic acid.

to morning. A one-hour control urine specimen was collected from 7 to 8 A.M. At 8 A.M. a fasting blood sample (control) was drawn, and then the subject swallowed 1 gm. of the glucuronolactone freshly dissolved in 25 ml. of water. Heparinized blood samples were obtained at one and three hours following ingestion of the lactone, and complete collections of urine were made at two-hour intervals for six hours. The subjects remained fasting but were allowed water during the experimental periods. The same procedure was used at later dates with 1 gm. D-glucuronic acid.

The dog used in this study was a male Boxer weighing 24.5 kg. The animal was given four separate intravenous infusions of 1 gm. of glucuronolactone and four intravenous infusions of 1 gm. of glucuronic acid. The animal was in a fasting state and was not anesthetized. On the morning of each test the bladder was catherized and a 200 ml. water load was given by stomach tube. After a half-hour control period of urine collection, a control blood sample was drawn and the animal was rapidly infused with 1 gm. of either the acid or lactone. Two half-hour periods followed, during which blood and urine urine samples were collected.

RESULTS

It was necessary to determine the effect of D-glucuronolactone, D-glucuronic acid and xylulose on the color development in the ascorbic acid method. It was found that 1 mg. of D-glucuronolactone gave a color equivalent of 0.005 mg. of L-ascorbic acid; 1 mg. of D-glucuronic acid gave a color equivalent of 0.05 mg. of L-ascorbic acid; 1 mg. of L-xylulose, although it formed the dinitrophenylhydrazone, when treated with the sulfuric acid gave no color at all; 1 mg. of D-glucose also gave no color.

Reductic acid, when assayed by this method, gave the same color equivalent as L-ascorbic acid. However, the chromatographic procedure readily differentiated the reductic acid and reductones from ascorbic acid.

Figure 1 demonstrates the mean results obtained when six human subjects ingested either D-glucuronolactone or D-glucuronic acid. When D-glucuronolactone was swallowed, the ascorbic acid level in plasma rose within one hour from a control of 0.39 (±0.30 S.D.) mg. per 100 ml. to 0.84 (±0.45 S.D.) mg. per 100 ml. and then fell to 0.62 (±0.46 S.D.) mg. per 100 ml. three hours after the administration of the D-glucuronolactone. These deviations were statistically significant when calculated as per cent change from control. Ascorbic acid levels in urine rose from a control of 2.25 (±0.96 S.D.) mg. per hour to 12.15 (±2.86 S.D.) mg. per hour during the first two-hour period following the administration of D-glucuronolactone. During the second two-hour period, the urine level dropped to 6.1 (±1.47 S.D.) mg. per hour and finally to 3.95 (±0.44 S.D.) mg. per hour during the third two-hour period. The mean milligram excess ascorbic acid excreted per hour during the six hours was 8 mg., whereas the milligram excess of ketopentose (xylulose) in urine was only 0.2 mg. per hour.

When the same test was performed using D-glucuronic acid instead of D-glucuronolactone, the ascorbic acid values in blood showed no significant increase from the control level. Also, the ascorbic acid values in urine did not show any marked change from the control level. The apparent milligram excess of ascorbic acid excreted during the test with D-glucuronic acid was only 0.6 mg. per hour. This level could be accounted for by

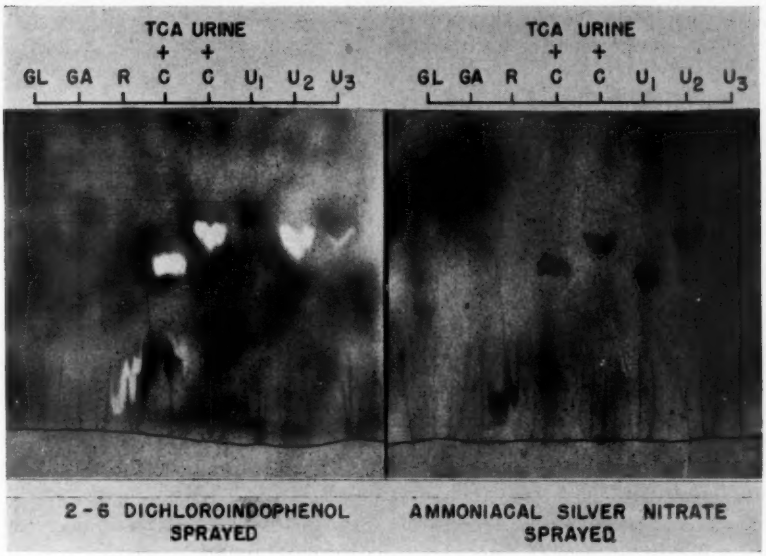

FIG. 2. A pair of partition diagrams demonstrating the presence and relative R_f values of reductic, ascorbic and dehydroascorbic acids. (For details see text.)

interference of the D-glucuronic acid in the chemical test. It is of interest that xylulose levels in urine rose from a control of 0.075 (±0.015 S.D.) mg. per hour to 10.3 (±1.10 S.D.) mg. per hour during the first two-hour period; this dropped to 5.25 (±0.63 S.D.) mg. per hour during the second two-hour period and then to 2.63 (±0.33 S.D.) mg. per hour during the third two-hour period. The milligram excess xylulose excreted by man during the tests with D-glucuronic acid averaged 8.1 mg. per hour.

The effects of 1 gm. infusions of D-glucuronolactone and D-glucuronic acid in the dog were similar (Fig. 1). After the dog was infused with the D-glucuronolactone, the mean ascorbic acid level in blood rose within 10 minutes from a control of 0.61 (±0.10 S.D.) mg. per 100 ml. to 0.96 (±0.10 S.D.) mg. per 100 ml. and in one hour fell to a level of 0.80 (±0.05 S.D.) mg. per 100 ml. The mean urine milligram excess ascorbic acid excreted by the dog was 10.3 mg. per hour, whereas the mean excess xylulose was only 0.25 mg. per hour.

The levels of ascorbic acid in blood and urine following the infusion of D-glucuronic acid matched those obtained with the D-glucuronolactone. In the blood the ascorbic acid level rose from 0.41 (±0.07) mg. per 100 ml. to 0.85 (±0.15) mg. per 100 ml. then fell to 0.53 (±0.06) mg. per 100 ml. one hour after the infusion. The excess ascorbic acid excreted during the experiments with D-glucuronic acid was 11 mg. per hour. The excess xylulose excreted during these same experiments was only 0.40 mg. per hour.

Chromatography revealed the presence of reduced ascorbic acid, as well as dehydroascorbic acid, in the urine of all subjects following the ingestion of D-glucuronolactone. However, reduced ascorbic acid could not be detected in their urine following the ingestion of D-glucuronic acid. Reduced ascorbic acid was not present in any of the control urine specimens, although traces of dehydroascorbic acid were present. The greatest concentration of reduced ascorbic acid appeared two hours after the ingestion of D-glucuronolactone and steadily decreased in proportion to dehydroascorbic acid until at the sixth hour the ascorbic acid present in the urine was almost wholly in the form of dehydroascorbic acid. This chromatographic test validated the results obtained using the ascorbic acid method of Shaffert

and Kingsley[5] when their chemical procedure was used to check the presence of both forms of ascorbate in several of the urine samples.

Chromatograms from a typical test with D-glucuronolactone in a human subject are illustrated in Figure 2. D-Glucuronolactone and D-glucuronic acid, when dissolved in 4 per cent trichloroacetic acid, did not react with the 2,6-dichloroindophenol, as can be seen by the total absence of white spots. Reductic acid (R) and ascorbic acid in the reduced form were indicated by white spots after spraying with indophenol. Pure ascorbic acid in urine (Urine + C) migrated more slowly than when dissolved in tricholoroacetic acid (TCA + C). The positions of the white spots of urine samples U_2 and U_3 have R_f values identical to the spot produced when pure ascorbic acid is added to control urine. The control urine sample, U_1, did not react with indophenol. However, when sprayed with ammoniacal silver nitrate, a light brown spot with an R_f of 0.41 was revealed, thus indicating the presence of dehydroascorbic acid. Following the ingestion of D-glucuronolactone, samples U_2 and U_3 indicated both dehydroascorbic acid, R_f 0.41, and reduced ascorbic acid, R_f 0.38, the latter having a rapidly developed black spot. The photograph also shows that most of the reduced ascorbate was excreted soon after the ingestion of the D-glucuronolactone. It was noted in other chromatograms that the R_f value of D-glucuronolactone was 0.64, of D-glucuronic acid 0.17, and of reductic acid, 0.82. However, these spots are difficult to see in Figure 2.

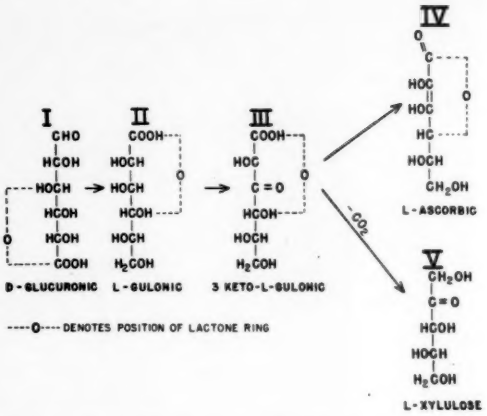

FIG. 3. Synthesis of L-ascorbic acid via D-glucuronic acid pathway.

COMMENTS

Bublitz[4] demonstrated that D-glucuronate can be transformed to L-gulonate and 3-keto-L-gulonate and then to L-ascorbate in rats, and showed that in certain cases 3-keto-L-gulonate could be decarboxylated to L-xylulose (Fig. 3). Burns[9,10] found no detectable incorporation of C^{14} from D-glucuronolactone or L-gulonolactone into L-ascorbic acid in guinea pigs *in vivo*, the values obtained being less than one-twentieth of those shown in rats. Lehninger et al.[11,12] reported no net synthesis of L-ascorbic acid from L-gulonolactone in human, monkey and guinea pig homogenates of liver; whereas significant synthesis of ascorbate acid occurred in rat, mouse, rabbit and dog homogenates of liver. Man does carry out the various steps required for the synthesis of ascorbate except for the conversion of the L-gulonate to ascorbate.[9] Therefore, it would be expected that glucuronic acid or glucuronolactone fed to man would be converted to the gulonate and then decarboxylated to L-xylulose. In the present experiments when glucuronic acid was fed, excess L-xylulose was excreted but little or no ascorbic acid, the reaction presumably proceeding *via* the decarboxylation of 3-keto-L-gulonate. When glucuronolactone, however, was fed, excess ascorbate was excreted with no change in xylulose excretion. The results suggest that man could convert lactonized L-gulonate to L-ascorbate. Bublitz and Lehninger[13] reported that rat liver enzymes converted L-gulonate to L-ascorbic acid from the lactone form but not from the free gulonate.

In the dog, which can synthesize vitamin C, both glucuronolactone and glucuronic acid could be converted to L-ascorbate. Little, if any, of either of the two compounds was diverted to xylulose.

Although these experiments suggest that man may synthesize ascorbic acid when given a proper precursor compound, they do not

exclude the possibility that the glucuronolactone displaced tissue ascorbic acid which passed into the blood stream and was excreted. It is also possible that the chemical and chromatographic procedures were detecting a "C"-like compound that was similar to the unidentified compound reported by Bublitz and Lehninger.[13]

SUMMARY

When human subjects were given 1 gm. of D-glucuronolactone orally the ascorbic acid levels in plasma increased. There was an excess renal excretion of 8 mg. per hour of ascorbic acid accompanied by an excess excretion of 0.2 mg. per hour of xylulose.

When the same subjects were given 1 gm. of D-glucuronic acid orally there was no increase in ascorbic acid levels in blood. Although no significant increase was noted in urinary ascorbic acid levels, the excess excretion of xylulose in the urine was 8.1 mg. per hour.

The infusion of a dog with 1 gm. of D-glucuronolactone or D-glucuronic acid caused an increase in the ascorbic acid levels in blood. The renal excretion of ascorbic acid was 10.3 mg. per hour during the experiments with D-glucuronolactone and 11 mg. per hour during the experiments with D-glucuronic acid. The xylulose excretion was not significantly changed.

REFERENCES

1. Horowitz, H. and King, C. Glucuronic acid as a precursor of ascorbic acid in the albino rat. *J. Biol. Chem.*, 205: 815, 1953.
2. Isherwood, F. A., Chen, Y. T. and Mapson, L. W. Synthesis of L-ascorbic acid in plants and animals. *Biochem. J.*, 56: 1, 1954.
3. Touster, O., Hutcheson, R. M. and Rice, L. The influence of D-glucuronolactone on the excretion of L-xylulose by humans and guinea pigs. *J. Biol. Chem.*, 215: 677, 1955.
4. Bublitz, C., Grollman, A. P. and Lehninger, A. L. Enzymatic conversion of D-glucuronate to L-ascorbate in animal tissues. *Fed. Proc.*, 16: 382, 1957.
5. Schaffert, R. R. and Kingsley, G. R. A rapid, simple method for the determination of reduced, dehydro-, and total ascorbic acid in biological material. *J. Biol. Chem.*, 212: 59, 1955.
6. Futterman, S. and Roe, J. H. The identification of ribulose and L-xylulose in human and rat urine. *J. Biol. Chem.*, 215: 257, 1955.
7. Baker, E. M., Bierman, E. L. and Plough, I. C. Factors influencing the urinary excretion of ketopentoses by normal men. *Metabolism*, in press.
8. Mapson, L. W. and Partridge, S. M. Separation of substances related to ascorbic acid. *Nature, London*, 164: 479, 1949.
9. Burns, J. J. Bio-synthesis of L-ascorbic acid. Basic defect in scurvy. *Am. J. Med.*, 26: 740, 1959.
10. Burns, J. J. and Evans, C. The synthesis of L-ascorbic acid in the rat from D-glucuronolactone and L-gulonolactone. *J. Biol. Chem.*, 223: 897, 1956.
11. Ul Hassan, M. and Lehninger, A. L. Enzymatic formation of ascorbic acid in rat liver extracts. *J. Biol. Chem.*, 223: 123, 1956.
12. Grollman, A. P. and Lehninger, A. L. Enzymatic synthesis of L-ascorbic acid in different animal species. *Arch. Biochem.*, 69: 458, 1957.
13. Bublitz, C. and Lehninger, A. L. The enzymatic conversion of L-gulonate to L-ascorbate by rat liver enzymes. *Biochim. et biophys. acta*, 32: 290, 1959.

Serum Vitamin B_{12} Concentration in Dietary Deficiency

JAMES A. HALSTED, M.D.,* JEAN CARROLL, B.S.,† ABBAS DEHGHANI, M.D.,‡
MEHDI LOGHMANI, M.D.‡ AND ANANDA S. PRASAD, M.D.§

MANY farmers in the villages of Iran are very poor, subsisting chiefly on a diet of whole wheat bread, tea and sugar. Some of them rarely, if ever, consume meat, chicken, eggs or other animal protein foods which are the source of vitamin B_{12}. Others are able to obtain these foods in small although regular quantities. The daily caloric intake from a diet consisting exclusively of wheat is estimated at approximately 2,400, with about 90 gm. of protein. However, vitamin B_{12} is not present in cereals or other vegetable products. The purpose of this investigation was to determine whether the serum vitamin B_{12} concentration was unusually low in these persons.

METHODS

Two groups of villagers were studied. The first group subsisted on a diet deficient in animal protein. They ate whole wheat bread almost exclusively with a small amount of milk product called mast, similar to yogurt, once or twice a week, and a little meat or chicken not more than once a month.¶ Many of this group had even less animal protein than this, some had none at all. The second group subsisted on a diet which contained meat, chicken or eggs as well as mast in regular amounts, at least every other day. There were twenty-three subjects in each group; all were patients in the Saadi Hospital, a free government institution. None had hepatic disease, renal insufficiency, gastrointestinal disease or leukemia, each of which might result in abnormalities in vitamin B_{12} metabolism and serum levels. The hemoglobin in all patients was at least 11 gm. per 100 ml. Blood samples were obtained at mid-morning, the serum being separated after clotting, and frozen. When all the samples were collected they were sent by air mail to Syracuse, New York, with an elapsed time of eight days. There the vitamin B_{12} concentration was determined using L. leichmannii as the test organism. The personnel and technic in this laboratory were the same as in a previous study of vitamin B_{12} serum concentration in various chronic disease states.[1] It was determined that the vitamin B_{12} level of serum frozen for ten days, then exposed to room temperature for ten days did not change significantly. This would simulate conditions obtaining in the present study, which was carried out during the winter months.

From the Nemazee Hospital, Shiraz Medical Center, the University of Shiraz Medical School, Shiraz, Iran, and the Veterans Administration Hospital, Syracuse, New York.

* Visiting (Fulbright) Professor of Medicine, University of Shiraz Medical Center, Shiraz, Iran. † Research Technician, Veterans Administration Hospital, Syracuse, New York. ‡ Medical Resident, Saadi Hospital, Shiraz, Iran. § Visiting Associate Professor of Medicine, University of Shiraz Medical School and Associate Physician, Nemazee Hospital, Shiraz Medical Center, Iran.

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¶ Five samples of wheat from different villages in the area were assayed for vitamin B_{12} using L. leichmannii. These samples showed no vitamin B_{12} activity but did show desoxyriboside activity of 55, 22, 55, 46 and 52 μg. per gm., respectively, in terms of thymidine. Because this desoxyriboside activity interferes with vitamin B_{12} activity in the L. leichmannii assay, the five samples were assayed with Ochramonas malhamensis. These assays showed no vitamin B_{12} activity in any of the samples.

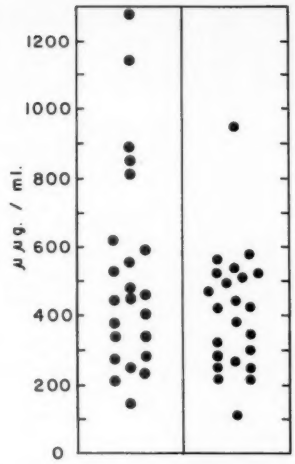

FIG. 1. Serum vitamin B_{12} concentration in twenty-three persons with an adequate diet (*left*) and twenty-three without an adequate diet (*right*).

RESULTS

Figure 1 shows the results of the serum vitamin B_{12} levels in the two groups. The mean level in the group with an "adequate" diet was 518 $\mu\mu$g. per ml. with a range of 133 to 1,300, and a standard deviation of 297. The mean level in the group with an "inadequate" diet was 411 $\mu\mu$g. per ml. with a range of 117 to 960 and a standard deviation of 167. There was no significant difference in the two groups ($t = 1.5$, $p = 0.1$–0.2).

COMMENTS

Although malnutrition is prevalent among the villagers of Iran who make up the bulk of the patient population of the Saadi Hospital, we have not seen any patient with megaloblastic anemia caused by dietary deficiency. The serum vitamin B_{12} concentration in these villagers who were the subjects of the present study did not differ significantly from that reported in 333 normal subjects from Syracuse, New York.[1] Furthermore, there was no significant difference between the group subsisting on a diet extremely low in animal protein from those whose diets contained adequate amounts. Animal protein is the only source of dietary vitamin B_{12}.

These findings indicate that vitamin B_{12} deficiency is not likely to occur even on a diet with extremely inadequate vitamin B_{12} intake. Three possibilities come to mind which might explain this situation

The first possibility is that the small amount of vitamin B_{12} present in the diet of the group on an inadequate intake was actually sufficient for the body requirement. This is estimated at 0.5 to 1 μg. per day. A group of extremely strict vegetarians known as Vegans has been studied from the standpoint of vitamin B_{12} deficiency in England. Although megaloblastic anemia was not observed, presumably due to a high folic acid content in their food supply, neurologic lesions and abnormally low serum vitamin B_{12} levels were present.[2]

A second explanation could be that the bacterial flora of the intestine in the patients studied might differ from the usual owing to the high carbohydrate cereal diet. Under these circumstances organisms capable of synthesizing vitamin B_{12} might ascend into the ileum. Although vitamin B_{12} is not absorbed from the colon it can be absorbed from the ileum.[3] Very little information is at hand respecting the bacterial flora of the small intestine. Although it is presumed to be sterile under normal conditions, the bacterial flora of the gut has not been studied adequately, especially in relation to various types of dietary intake. Some bacteria are known to synthesize vitamin B_{12}, and others require it for their growth. For example, Foy et al.[4] have found that non-addisonian megaloblastic anemia which occurs in Africans on inadequate diets may repond to penicillin given orally. These subjects have very low serum vitamin B_{12} levels. They speculate that in these subjects the diet encourages the growth of bacteria competing for vitamin B_{12}, but that other subjects without anemia depend on bacterial synthesis for their supplies of vitamin B_{12}, folic acid and perhaps some essential amino acids.

The third possible explanation for the lack of vitamin B_{12} deficiency in the presence of such inadequate diets is that the villagers literally live with their animals (donkeys, cows, sheep and goats), because they are driven into the village compound at night. The yards of the village are constantly littered with manure

and it is not difficult to imagine that the inhabitants might ingest a certain quantity of it through such close contact. Feces of ruminant animals contains large amounts of cobalamins including cyanocobalamin (vitamin B_{12}).

SUMMARY

Serum vitamin B_{12} concentrations in twenty-three villagers of Iran consuming a diet extremely low in animal protein and presumably in vitamin B_{12} content did not differ significantly from that found in twenty-three villagers who ate animal protein and who presumably received adequate amounts of the vitamin. Possible explanations are offered for this finding. The serum vitamin B_{12} concentrations were not significantly different from those found in normal persons in the United States. This study indicates that vitamin B_{12} deficiency caused only by a deficient diet must be very rare.

ACKNOWLEDGMENT

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REFERENCES

1. HALSTED, J. A., CARROLL, J. and RUBERT, S. Serum and tissue concentration of vitamin B_{12} in certain disease states. *New England J. Med.*, 260: 575, 1959.
2. WOKES, F., BADENOCH, J. and SINCLAIR, H. M. Human dietary deficiency of vitamin B_{12}. *Am. J. Clin. Nutrition*, 3: 375, 1955.
3. CITRIN, Y., DEROSA, C. and HALSTED, J. A. Sites of absorption of vitamin B_{12}. *J. Lab. & Clin. Med.*, 50: 667, 1957.
4. FOY, H., KONDI, A. and MANSON-TSAHR, P. E. C. Penicillin in megaloblastic anemia of Africans. Effect on serum vitamin B_{12} levels and absorption of radioactive vitamin B_{12}. *Lancet*, 2: 693, 1955.

Diet Therapy

The Role of the Food and Drug Administration in Nutrition

GEORGE P. LARRICK, D.SC.*

EVERY physician, dietitian and nurse, indeed, everyone concerned with nutrition, should know what the Food and Drug Administration does in nutrition and related areas of interest to the consumer. Standards for "enriched" foods, nutritional "quackery," safety of food additives, "tolerances" for pesticide residues on food crops—these are phrases which only comparatively recently have come into our vocabulary. Such phrases are loaded with implications for the safety, wholesomeness, nutritional quality and integrity of foods for both general and special dietary use. They are, of course, of special interest to nutritionists. This is not only a matter of professional competence, but also involves an obligation to know the strengths and weaknesses of our protective laws, and to be alert to both necessary and undesirable changes which may be proposed.

As an introduction to the work of the FDA in this area, let us look briefly at the broad pattern set forth in the Federal Food, Drug, and Cosmetic Act of 1938.

THE BREADTH OF CONSUMER PROTECTION

This law is a revised and greatly strengthened version of the original Federal Food and Drugs Act of 1906. Dr. Harvey W. Wiley, Chief Chemist of the U. S. Department of Agriculture at the turn of the century, deserves a generous share of gratitude for his crusade on behalf of the original act. The major objectives of the current law are sometimes summarized as:

Safe, effective drugs and cosmetics.
Pure, wholesome foods.
Honest labeling and packaging.

The law forbids shipment in interstate commerce of "adulterated" or "misbranded" foods, and defines adulteration and misbranding to accomplish its purpose of protecting consumers. Violators may be fined or imprisoned, or both, and illegal goods may be seized to remove them from the market. All actions must be taken through Federal district courts. The court may also issue an "injunction" to prevent repetitious violations or any violation which appears imminent.

IMPORTANT DEFINITIONS OF ADULTERATION AND MISBRANDING

A frequent concept of the meaning of "adulteration" is simply the addition of a substance to cheapen the article. While the law includes this concept in its definition, it goes much beyond this. Some of the important definitions of *adulteration of food* contained in the law are these:

Preparation, handling or storage under unsanitary conditions.

Presence of filthy, putrid or decomposed material.

From the Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, D. C.

* Commissioner of Food and Drugs.

Presence of any substance which would make the food injurious to health.

Presence of any residue from pesticide or food additive not generally recognized as safe, except as may be permitted by specific regulation setting a limit (tolerance) on the amount which may be present.

Presence of any coal-tar color not listed by the FDA as harmless and tested for purity in laboratories of the FDA.

Omission of an important ingredient, or substitution of a cheaper ingredient, or use of an ingredient to make the food appear better than it is.

Some of the important definitions of *misbranding of food* are:

False or misleading statements on the label or in labeling.

Deceptive packaging or shortage in net contents.

Failure to label the food as an imitation, if it is an imitation of another food.

Omission of special dietary information needed by users (as, for example, the sodium content of foods sold for use in low-sodium diets).

Failure to conform to any standard of identity (composition), quality or fill of container set by regulation.

Omission of the information required by law to be on the label: the common or usual name of the food and of each ingredient, with certain exceptions; the name and address of the manufacturer, packer or distributor; an accurate statement of contents; and declaration of the presence of any artificial flavor, artificial color or chemical preservative.

The law, of course, contains other definitions of adulteration of food and misbranding, but they all add up to require that a food be safe, clean, wholesome *and what it purports to be, both in quality and quantity*. There are also, of course, special definitions for adulteration and misbranding of drugs and cosmetics.

PREMARKETING CONTROL FOR SPECIAL PRODUCTS

Most consumers, and perhaps many doctors and nutritionists, do not understand that special *premarketing* controls are provided for a few special classes of products only; and that for the great bulk of foods, drugs and cosmetics the law operates through periodic inspection of factories and sampling of products after they are on the market.

The following is a list of products for which there is premarketing control, and the type of control provided for each:

New drugs: manufacturers must submit experimental proof of safety under conditions of proposed use.

Antibiotics (penicillin, streptomycin, chlortetracycline, chloramphenicol and bacitracin and their derivatives, only) and insulin: manufacturers must submit proof of both safety and efficacy; and each batch must be tested in FDA laboratories for purity and potency before it may be distributed.

Pesticides: manufacturers must submit experimental proof of the amount of residue that will remain on crops if directions on the label are followed; and that this amount of residue will not be harmful. The FDA then sets a safe "tolerance" for each crop, and this amount of residue must not be exceeded on the food as shipped.

Food additives: manufacturers must submit proof of safety of the amount to be used, and of technical effect or benefit; the FDA sets safe tolerances for use in specific foods, and specifies any other conditions of use necessary to protect the health of the public.

Coal-tar colors: the color must be one listed by the FDA as "harmless and suitable for use;" and each batch must be tested for purity in FDA laboratories. (With one exception covered by special legislation, the law does not permit the FDA to limit the amount of a listed coal-tar color. Some colors originally believed harmless have recently had to be removed from the list of permitted colors, because new tests have shown that large amounts will cause injury to animals. An amendment to the law is being sought to permit the FDA to set safe limits on the amount of a color which may be used.)

For products in these classes, marketing without advance clearance by the FDA, or except in compliance with the terms of that clearance, is *per se* a violation of the law.

To review the manufacturers' experimental evidence of safety of new drugs, food additives,

TABLE I

Minimum Daily Requirements of Vitamins Established for Labeling Purposes

Age Group	Vitamin A (USP Units)	Vitamin D (USP Units)	Vitamin C (USP Units)	Thiamine (mg.)	Riboflavin (mg.)	Niacin (mg.)
Adults	4,000	400	30	1.0	1.2	10
Children (all ages)	3,000	400	20	...	0.9	...
6 yr. or older	...	...	..	0.75	...	7.5
Less than 6 yr.	...	...	..	0.5	...	5
Infants	1,500	400	10	0.25	0.6	...

and pesticides, the FDA has a headquarters staff composed of some of the country's leading scientists, including physicians, pharmacologists, chemists, nutritionists, bacteriologists and workers in related fields. These scientists not only must pass judgment on the adequacy of the work done by the manufacturers, but also must conduct or direct their own studies where necessary to check on doubtful points. On their advice the Commissioner of Food and Drugs determines whether or not these products may be marketed.

These scientists also develop highly specialized laboratory procedures needed by the field laboratories of the FDA to detect and prove violations of the law; they also make certain types of assays—for example, of certain vitamin products and bioassy drugs—which cannot be conducted in the field laboratories. The FDA's Division of Nutrition also conducts certain special investigations which will be of interest to the reader, and which will be mentioned later.

ALL OTHER PRODUCTS POLICED BY FACTORY INSPECTION AND MARKET SAMPLING

For all products other than those listed above, the policing of purity and safety begins with scientifically trained inspectors and scientists in seventeen field laboratories in major cities throughout the country.

The 430 Food and Drug Inspectors of the FDA are not only experts in the location of any defects in a food, drug or cosmetic factory operation which may result in an inadvertent violation of the law but also in the detective work necessary to catch the occasional deliberate violator. Sanitation in plants, manufacturing processes, suitability of raw materials, labeling and packaging practices and control in plants to guard against mistakes—all are subject to the inspector's strictest scrutiny. Refusal of a manufacturer to permit inspection is a violation of the law.

Most manufacturers value highly the inspector's comments on their operations to help them keep out of trouble; but, of course, if a violation is found, the inspector's report sets in motion a series of actions which may end up in the Federal court.

Backing up the inspectors are trained chemists and other scientists in the seventeen FDA District Laboratories, who examine samples from interstate shipments to detect any violation of the law. These scientists may develop evidence to support what the inspector has observed, or they may find evidence of a violation which was concealed from the inspector, which could not be detected except by laboratory analysis.

Inspectors of the FDA can examine the nearly 90,000 food, drug and cosmetic establishments subject to the Federal law on an average of about once every three to four years. A Citizens Advisory Committee which studied the Food and Drug Administration in 1955 believed the long interval between inspections to represent a serious defect in consumer protection. A three to fourfold expansion of the 1955 staff of approximately 250 inspectors was recommended, and considerable progress has been made toward this goal. The increase of $2,883,000 in our appropriation for 1960 will enable us to catch up with the minimum rate of progress recommended by the Committee, which called for completion of the growth within a five-to ten-year period.

TABLE II
Minimum Daily Requirements of Minerals Established for Labeling Purposes

Age Group	Calcium (mg.)	Phosphorus (mg.)	Iron (mg.)	Iodine (mg.)
Adults	750	750	10	0.1
For pregnancy and lactation	1,500	1,500	15	...
Children (all ages)	750	750	...	0.1
6 yr. or older	...	...	10	...
Less than 6 yr.	...	...	7.5	...

FDA PROGRAMS OF SPECIAL INTEREST TO NUTRITIONISTS

Vitamin- Mineral Products

The law requires informative labeling on foods purporting to have value for special dietary uses. For example, foods claiming to be of special value as dietary supplements because of their vitamin or mineral content must specify the amount of each vitamin or mineral on which such value is based, in terms of the proportion of the minimum daily requirement supplied by a quantity of the food which might reasonably be eaten in a day.

For this purpose minumum daily requirements for six vitamins and four minerals are designated by regulation. Tables I and II list these values.

The requirements shown are amounts of the vitamins and minerals that are adequate to prevent deficiency and to allow for normal physiologic functions of the human body. These values were established in a wide variety of experimental and clinical studies. The same body of scientific information was the basis for the Recommended Dietary Allowances published by the Food and Nutrition Board of the National Research Council which are somewhat greater than the minimum daily requirements. The most recent National Research Council bulletin (Publication 589, 1958) entitled "Recommended Dietary Allowances" explains that the allowances for vitamins and minerals cited are liberal since they are meant to afford a margin of sufficiency above minimum requirements, are widely used in the planning of diets, and are designed to maintain good nutrition in all healthy persons under all variations in the population at large.

The differences between the two sets of values are not great, except for ascorbic acid, where the Recommended Daily Allowance is two and a half times the Minimum Daily Requirement. It must be recognized, however, that frequent reference to both "M.D.R." and "R.D.A." in the labeling of vitamin-mineral products presents a likelihood of consumer confusion, and suggests the desirability of a single set of authoritative values.

Other Foods for Special Dietary Uses

An artificial sweetener, or a food in which it is used in place of sugar, must state on its label that it is for use only by persons who must restrict their intake of ordinary sweets. A so-called "low-calorie" food must state the number of calories in a specified quantity, and if the calorie content is not significantly lower than that of other foods, "low-calorie" claims are considered false and misleading.

Foods purporting to be of value for low-sodium diets must state the number of milligrams of sodium per 100 grams of food, and per average serving.

The FDA examines samples of dietary supplements and other foods for special dietary uses to check the accuracy of the composition declared on the label, and to see that no false or misleading claims are made.

Food Standardization and Enrichment

The FDA is authorized to issue regulations setting standards of identity, quality and fill of container for processed foods. Industry, consumers, dietitians and nutritionists, and other interested persons may participate in the standards-making process. Consumers, dietitians and nutritionists, for example, might contribute information as to what consumers expect the food to contain. A standard must promote honesty and fair dealing in the interest of consumers.

A public hearing is held on any controversy as to what the standard should be. The standard as finally issued must be based on the record of the hearing, or on the facts of record submitted by interested parties.

For example, some of the standards issued to date:

Set minimum fat and maximum moisture for many cheese and cheese products and for oleomargarine.

Set minimum oil content for salad dressings.

Set minimum tomato solids content for tomato ketchup, tomato juice and tomato paste.

Set minimum fruit content for many jams, jellies and preserves.

Specify permissible ingredients and maximum moisture content for flours, breads and rolls.

Specify minimum quality factors for many processed canned fruits and vegetables.

Specify the amount of food which must be in the container for many processed canned foods.

Standards have been established for a number of "enriched" foods, where it has been determined that the food is an appropriate carrier for the enrichment ingredients, and the general nutritional health would be served thereby. Federal standards do not require any basic food to be enriched; but they require that if the food is to be labeled as "enriched" it must contain the amounts and kinds of added enrichment ingredients called for by the standard, and no others. This saves the consumer the confusion which would result from a multiplicity of different types of enrichment for the same basic food, and the variety of unwarranted claims which would likely be made for them.

The FDA checks standardized foods, and particularly the enriched foods, to see that they comply with the standards.

Nutritional Quackery

During the past two decades the public has become increasingly nutrition-conscious, and particularly vitamin-conscious. This fact has been exploited by the promoters of the so-called health foods and the peddlers of certain vitamin-mineral type supplements. Despite the belief of most authorities that the *average* diet of a healthy American does not need supplementation with vitamins and minerals, it is now estimated that Americans spend $500 million a year for these products! This is due in large part to the lecture-hall pitchmen and door-to-door peddlers who hold their products out as practically cure-alls for the ills of mankind. While the products usually are not harmful in themselves, there is a danger that seriously ill persons may be led by these promoters to delay needed medical treatment, with the result that their conditions become more difficult, or perhaps impossible, to treat successfully.

The Food and Drug Administration is waging major campaigns, both educational and enforcement, against nutritional quackery. The American Medical Association, American Dietetic Association, and the National Better Business Bureau have joined in the educational campaign. The FDA has brought many court actions against false and misleading label claims for these products, and against products which were extravagantly promoted orally. But these cases are time-consuming and the FDA staff cannot deal adequately with the problem. Public education to dry up the market for over-promoted products not needed for supplementation of the adequate diet would seem to be the ultimate answer.

Nutritional Research

Several years ago a well known commercial infant formula was found to be causing convulsions in children for whom it was used as the complete diet. The product was recalled from the market, the formula changed on the basis of research by the company and marketing resumed. The illnesses occurred again, and again the product was withdrawn, the formula changed and the cycle repeated. Meantime, FDA scientists in the Division of Nutrition were busy with the problem. Working on a lead from earlier research only remotely related, an FDA scientist suggested that the commercial formula might be deficient in vitamin B_6, and that it was this deficiency that caused the illnesses reported. It was then demonstrated clinically[1] that injection of vitamin B_6 brought almost immediate recovery of infants in convulsion. Vitamin B_6 was added to the formula, and no further illnesses occurred.

More recently, a mysterious outbreak of disease in flocks of poultry caused the loss of several million birds on the East Coast and in the Middle West. Scientists in university and

commercial laboratories determined that the illnesses of poultry were caused by poultry feed containing a black, tarry residue left after several distillations of fat to obtain fatty acids.[2]

In order to determine whether a violation of the law had occurred, FDA scientists took up the investigation at this point. They have isolated experimentally a portion of the black tarry residue which will cause the disease in poultry and a different portion which is definitely toxic to rats. Investigation of the physiologic effects of the constituents of heated fats is being continued.

Research projects on requirements for vitamins and minerals, amino-acid composition and protein quality of food, relation of blood cholesterol to dietary changes, and utilization of various carbohydrates are examples that illustrate how law enforcement activities often contribute to nutritional and medical progress beyond the objective of law enforcement.

The Food and Drug Administration will welcome additional participation by physicians, nutritionists and dietitians in discussions of nutritional problems which arise in our law enforcement work.

CONCLUSION

Thus, the protection of the nutritional quality of our natural food supply is a part of the basic design of the Federal Food, Drug, and Cosmetic Act. The administration of the law to accomplish its objective becomes increasingly difficult and at the same time increasingly important as modern food technology develops new products and processes to meet the demands of our progressive society. Law enforcement can be no better than the scientific knowledge underlying it. Science must develop the facts with which to combat pseudoscience, and consumer education becomes more and more important. The Food and Drug Administration must have the support of the professional nutritionist if it is to be most effective in performing its own role in nutrition.

REFERENCES

1. Nelson, E. M. Association of vitamin B_6 deficiency with convulsions in infants. *Pub. Health Rep.*, 71: 445, 1956.
2. Friedman, L., Firestone, D., Horwitz, W., Banes, D., Anstead, M. K. and Shue, G. Studies of the chicken edema disease factor. *J. A. Official Agric. Chem.*, 42: 129, 1959.

Reviews of Recent Books

Human Nutrition and Dietetics, by Sir Stanley Davidson, A. P. Meiklejohn and R. Passmore. Williams & Wilkins Co., Baltimore, 1959, pp. 816, $15.00.

The British have long been leaders in the field of nutrition. They have also paid particular attention to food processing and matters of public health. This book, written by three authorities from the University of Edinburgh, reflects what might be called the British approach to nutrition.

As noted in the preface, the book is intended for physicians, dietitians, food technologists and public health workers, all of whom need at least a working knowledge of the problems of human nutrition. Then follows a general description of chemical and nutritional properties of foods. The third section deals with diseases primarily associated with nutritional defects while the fourth part deals with the use of diet therapy in many medically important diseases. Nutrition in relation to public health and the role of certain international agencies is the topic of the next section. Finally, part six deals with the special problems of pregnancy, lactation, athletic training and climatic extremes.

In this wide survey of a highly complex field it is inevitable that some topics must be described only briefly. Nevertheless, a remarkably wide range of topics is covered in this book. Several minor criticisms can be mentioned: there is no discussion of the Schilling test on the section on vitamin B_{12} and nothing about the work of Holt and Snyderman on induced pyridoxine deficiency in infants. The paragraph on glucagon contains several errors and omissions. One particularly worthwhile feature is the generous use of photographs of such diverse topics as a subject using a Douglas bag, a sorghum granary in the Sudan, a rachitic dog and hunger victims of a concentration camp. There are numerous references but these tend to be the pioneering or "typical" papers and may not always be especially useful to the general reader. Thus in the discussion of experimental avitaminosis A, there is mention of Wolbach's early work (1925) but no reference to Warkany's more recent studies. Nevertheless, most current views are adequately presented.

The authors have set for themselves the difficult task of writing a book "for anyone interested in applying modern scientific knowledge to the practical problems of human nutrition." This includes, as they note, people in many different walks of life. It is to be expected, therefore, that readers of diverse backgrounds will find this volume useful and informative. Similarly it cannot be all things to all readers and some may wish for more detail in certain sections compared to others. The book deservedly will fill a need for a comprehensive view of nutrition. S. O. W.

Metabolic Care of the Surgical Patient, by Francis D. Moore. W. B. Saunders Co., Philadelphia, 1959, pp. 1,011, $20.00.

This is a unique book. The author, Professor of Surgery at Harvard Medical School, has been a leader in the school of surgical physiology, which has brought nutrition and metabolism from the laboratory to the bedside. Much of our present knowledge on shock, starvation, dehydration, electrolyte imbalance and stress reactions is due to this approach. Thus, a long, detailed analysis of common surgical problems by an active investigator may be expected to be authoritative, and this book is.

Not only is a monumental mass of material presented, but the organization of the book is refreshingly unusual. The work is divided into six sections, each with its own table of contents, systematic discussion, "notes" (summaries) from the literature, illustrations and case records—"individual surgical stories that illustrate a variety of points." In addition there are well over 1,500 references, 143 clear figures and an appendix featuring surgical diets and parenteral supplements.

Although the author has limited himself to areas of surgical care with which he is personally familiar, almost every topic of any importance is covered. Of widest interest is the first section dealing with endocrine and nutritional aspects of convalescence.

It can be stated without fear of contradiction that this major work is necessary reading for *all* surgeons in whatever specialty and highly recommended for all physicians who want a broad integrated view of pre- and postoperative management from the modern metabolic approach. S. O. W.

Iron Deficiency Anaemia. Report of a Study Group. World Health Organization Technical Report Series No. 182, 1959, pp. 15, $.30.

Iron deficiency anemia is a public health problem of considerable importance in the underdeveloped and tropical areas of the world. A WHO Study Group reviewed present knowledge of this condition and outlined further work that might be done to study the incidence, etiology and prophylactic measures that might be instituted. There appears to be no appreciable

deficiency of iron in most tropical diets. However, the *type* of diet consumed seems to result in decreased absorption of iron. Other factors which upset iron balance are increased losses in sweat and losses of blood caused by hookworm infection. An adequate intake of protein might also be associated with the production of this form of anemia. W. H.

Therapeutic Nutrition with Tube Feeding, by M. D. Pareira. Charles C Thomas, Springfield, Ill., 1959, pp. 58, $3.75.

This short book was written by the Director of Surgery, Jewish Hospital of St. Louis. In essence it is a documented review article and features twelve tables and seventeen figures. The principles and practices of tube feeding in surgery are presented succinctly and the author has obviously had much experience. Thus the information is readily applicable in the clinic.

Although this reviewer can find no fault with the material *per se*, he wonders why a short paper is converted into a hard-covered relatively expensive book. As far as the material goes, it will be welcomed, particularly by surgical house officers. S. O. W.

Arteriosclerosis and Nutrition (Arteriosklerose und Ernährung, Wiss. Veröffentl. d. Dtsch. Ges. f. Ernährung, Bd. III). Verlag Dr. Dietrich Steinkopff, Darmstadt, 1959, pp. 246, DM 45.

The book contains fourteen papers (with stenographic records of discussions) given at the 2nd Symposium of the German Society for Nutrition, Bad Neuenahr, October 17–18, 1958, under the chairmanship of Prof. Dr. H. W. Bansi of Hamburg. The participants are well known investigators from Germany, Austria, Sweden, Holland and Hungary. Many aspects of arteriosclerosis were covered, such as pathologic anatomy, biochemistry, albumin-bound carbohydrate, serum lipoproteins, fat metabolism, polyunsaturated fatty acids and statistical evaluation of data on relationship between nutrition and arteriosclerosis.

The book will be helpful to those who read German in getting some insight into the research activities in the field of arteriosclerosis in the European countries. Two papers would seem to be of special interest to American investigators. Professor Fritz A. Pezold of Free Berlin University reviewed the data obtained from 6,507 autopsies at the University's hospital in the years 1947 through 1949 and 1955 through 1957. In the former period, during which the population in West Berlin had been suffering from malnutrition since 1944, severe coronary atherosclerosis was found in 1,554 of 3,045 patients (51 per cent). In the latter period, in which there was excessive food consumption, the lesions occurred in 2,720 of 3,462 patients (78 per cent). Drs. K. Jahnke and A. Breitbach of Düsseldorf University presented detailed data on the average daily dietary intake in the period from 1932 to 1957 in Germany and in Düsseldorf. In terms of calories from fat, carbohydrate, protein and total, the intake was 85, 1,010, 200 and 1,295, respectively, in 1947; and 1,130, 1,740, 330 and 3,200, respectively, in 1957. There was a close correlation between the morbidity and mortality rates of coronary atherosclerosis and food consumption, particularly fat consumption. G. C. Chiu

Books Received

Books received for review by The American Journal of Clinical Nutrition are acknowledged in this column. As far as practicable, those of special interest are selected, as space permits, for a more extensive review.

Manual of Applied Nutrition of the Johns Hopkins Hospital, by Janette Carlsen. The Johns Hopkins Press, Baltimore, 1960, pp. 134, $3.00.

Modern Nutrition in Health and Disease, 2nd edition, by Michael G. Wohl and Robert S. Goodhart. Lea & Febiger, Philadelphia, 1960, pp. 1,152, $18.50.

Vitamins and Hormones. Advances in Research and Applications, Volume XVII, edited by Robert S. Harris, G. F. Marrian and Kenneth V. Thimann. Academic Press, Inc., New York, 1959, pp. 359, $14.00.

Vitamin B_{12} Metabolism. Some Studies on the Absorption, Excretion, Enterohepatic Circulation, Turnover Rate, Body Distribution and Tissue-Binding of B_{12}, by Peter G. Reizenstein. Gummessons Boktryakeri Stockholm, 1959.

Introduction to Foods and Nutrition, by Gladys T. Stevenson and Cora Miller. John Wiley & Sons, New York, 1960, pp. 517, $6.25.

Ciba Foundation Study Group No. 3. Cancer of the Cervix. Diagnosis of Early Forms, edited by G. E. W. Wolstenholme and Maeve O'Connor. Little, Brown & Co., Boston, 1959, pp. 114, $2.50.

Diabetes. With a Chapter on Hypoglycemia, edited by Robert H. Williams. Paul B. Hoeber, Inc., New York, 1960, pp. 793, $20.00.

Infant Foods and Feeding Practice, by Herman F. Meyer. Charles C Thomas, Springfield, Ill., 1960, pp. 332, $9.75.

Ciba Foundation Colloquia on Ageing. Volume 5. The Lifespan of Animals, edited by G. E. W. Wolstenholme and Maeve O'Connor. Little, Brown & Co., Boston, 1960, pp. 324, $9.50.

Ciba Foundation Symposium: Biochemistry of Human Genetics, edited by G. E. W. Wolstenholme and Cecilia M. O'Connor. Little, Brown & Co., Boston, 1960, pp. 347, $9.50.

Ciba Foundation Tenth Anniversary Symposium on Significant Trends in Medical Research, edited by G. E. W. Wolstenholme, Cecilia M. O'Connor and Maeve O'Connor. Little, Brown & Co., Boston, 1960, pp. 356, $9.50.

Klinische Physiologie. Aktuelle Probleme in Übersichten, by W. A. Müller. Georg Thieme Verlag, Stuttgart, 1960, pp. 118, DM 23 ($5.50).

Les Oedemes. Physiopathologie et Traitment de la Rétention de Sel et d'Eau, by Jean Fabre. Masson et Cie, Paris, 1959, pp. 344, 55.00 NF.

Abstracts of Current Literature

CHARLES R. SHUMAN, M.D., EDITOR

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JOHN WATERLOW, M.D., *Kingston, Jamaica*

DENTAL CARIES

The national cost of restoring teeth and correcting dental defects is enormous and represents a big item in many family budgets. Dental caries have been attributed to inheritance, metabolic disturbances, local conditions in the mouth, including composition of saliva and types of food consumed and the lack of fluorine in water supplies. The role of diet in formation of caries has been widely investigated with results which are conflicting, possibly due to the many factors involved in cariogenesis. Good dentition is of primary importance in good nutrition.

Caries in the Otomi Indians of Mexico. H. H. Neumann and N. A. DiSalvo. *New York Dent. J.*, 24: 63, 1958.

The incidence of caries in the Otomi Indians is described as being only one-tenth to one-hundredth as high as that of persons living in modern urban communities with the usual availability of "industrialized" foods. The food habits of the Otomi are monotonous and the diet is limited to a few basic items, chiefly corn as tortillas providing 70 to 80 per cent of the caloric requirement, broad beans and pulque, the fermented juice of the agave. The consumption of dairy products and meat is low. A variety of "weeds," worms and insects are consumed to some extent. In two villages, twenty-four adult males had an average of 0.6 decayed, missing and filled (DMF) teeth. In another larger community where more modern food items were available, thirty-four Otomi Indians had an average of 1.5 decayed, missing and filled teeth. In a very remote village, nineteen adult males were essentially caries-free with only one cavity in the whole group and no missing or filled teeth.

The fluoride content of the well waters in this area varied from 0.1 to 0.3 with no evidence whatever of dental fluorosis among the Indians. The diets of the Otomi were largely composed of soft and liquid materials. However, the teeth were used for a variety of tasks requiring vigorous use of the masticating apparatus: cracking bones of small animals to extract the marrow, opening two kinds of hard-shelled nuts that form a frequent part of the diet and the chewing of toasted "haba" beans and toasted whole corn. Dynamometer readings of maximum jaw force indicated that these Indians had much higher abilities to exert jaw force than did American athletes.

The authors postulate that the keratin in the enamel may react to vigorous chewing forces much as the epidermis of the skin reacts to increased physiologic stresses and that this keratin may have a greater ability to protect the hydroxyapatite crystals of the teeth. These postulates represent a departure from prevailing oral environmental concepts of the etiology of dental caries and suggest the need for further exploration to determine whether such a biophysical influence occurs in teeth. J. H. SHAW

Caries in Indians of the Mexican Cordillera, the Peruvian Andes and at the Amazon Headwaters. H. H. Neumann and N. A. DiSalvo. *Brit. Dent. J.*, 104: 13, 1958.

The relationships of food habits and the nutritional quality of the diet to the incidence of dental caries in population groups have often been explored and various hypotheses about the etiology of dental caries based thereon. In the present survey, the food habits and the biting force of Indians in a series of eight tribes in fifteen villages in Mexico, Guatemala and Peru were studied. The lowest average number of decayed, missing and filled teeth per person was observed to

be 0.05 among nineteen males in an Otomi village in Mexico. The highest incidence was 3.3 among twenty-four males in a Shipibo village in Peru.

Generally, the diets were high in carbohydrates and low in protein and fat with few of the foods ordinarily considered essential in balanced nutrition. Among the Otomi Indians, there was evidence of riboflavin and niacin deficiencies with clinical signs of angular cheilosis and glossitis. In other tribes there was evidence of borderline intakes of calcium, vitamin A, riboflavin and ascorbic acid and of animal proteins. Major differences were observed in the amount of chewing required by the tribal diets. Although many staple foods were soft or liquid in form, numerous foods required unusual amounts of biting force, such as the "haba" bean and the cracking of bones and nuts. In tests with a gnathodynamometer which measured the maximum force that the subject could exert with his masticatory apparatus, the tribes with the lowest average incidence of dental caries had, by and large, the highest average dynamometric readings of biting force. Likewise, where the incidence of caries was high there was a minimum requirement for vigorous chewing.

The authors felt that the relationship of diets to dental caries among these Indian tribes was a biophysical rather than a biochemical one. They postulated that the degree of chewing may influence the molecular structure and ionic exchange of the teeth in such a way that vigorously used teeth become thereby inherently more resistant to the oral environmental impetus toward the production of carious lesions.

J. H. SHAW

Trace Elements in Ancient Indian Teeth. L. T. Steadman, F. Brudevold, F. A. Smith, D. E. Gardner and M. F. Little. *J. Dent. Res.*, 38: 285, 1959.

Analyses were made for carbonate, fluoride, zinc, silicon, manganese, lead, silver, copper, tin, strontium, iron and aluminum on twelve Pueblo Indian permanent teeth discovered in Pueblo Bonito, Mexico, after burial for approximately 800 years and twenty-three Indian Knoll permanent teeth excavated from the bank of Green River in Kentucky after burial for approximately 5,000 years. Analyses were made on successive layers of the enamel and the roots which had been prepared by grinding over a porcelain filter. Among the Pueblo Indian teeth all the trace elements included in the analyses occurred in greater concentrations in the outer than in the inner enamel, whereas the carbonate concentration increased from the outer to a maximum in the deepest layer of enamel. In the external enamel, fluoride occurred in the greatest concentration, followed by zinc, silicon, manganese, lead, silver, copper and tin. The concentration of zinc varied less from the outer to the inner surface of the enamel than any of the other trace elements. In the roots of these same teeth, the various trace elements again occurred in greater concentration in the surface than in the subsurface layers, with the exception of manganese, for which a fairly uniform distribution was noted.

Among the Indian Knoll teeth, the distribution of trace elements was generally similar to that in the Pueblo Indian teeth. However, the accumulation of fluoride, silicon and manganese on enamel surface was more marked among the Indian Knoll samples; the Pueblo Indian teeth contained greater amounts of zinc, silicon, manganese and silver but less copper than the Indian Knoll teeth. The dentin in the Pueblo Indian teeth had much greater concentrations of fluoride, zinc, silicon and manganese than enamel but this ratio was much less for the Indian Knoll teeth. In comparison with contemporary teeth, the distribution of these trace elements was remarkably similar to that in these ancient Indian teeth. This finding was contrary to expectation and indicated that a much higher percentage of trace elements is incorporated into teeth during life from the foods consumed than is acquired during centuries of interment.

J. H. SHAW

Diet and Dental Health in Newfoundland Children. T. Y. Lin and M. D. Smith. *Canad. J. Pub. Health.*, 49: 516, 1958.

In the present study the incidence of dental caries and the quality of the diet are compared. The total number of teeth in the mouth, the decayed and missing teeth and the number of decayed tooth surfaces were noted as recorded for each of 400 children. These data were expressed as the "caries figure" which is the ratio of caries to the total number of teeth in the mouth, i.e., the average number of decayed tooth surfaces per tooth. The dietary information of the families as given by the mothers was noted for each factor studied as "daily," "frequently," "irregularly," "seldom" or "never" under the following headings: breast feeding, weaning formula, vitamin C supplement, vitamin D supplement, milk, citrus fruits, other fruits, vegetables, potatoes, meat, liver, fish, eggs, bread, cereal, cheese, pastry and candy. Arbitrary points were given to these various factors on the basis of the authors' conception of their relative importance: the poorest diets had nutrition scores by this system of 0 to 3, the fair diets 4 to 7, the average diets 8 to 11 and the good diets 12 to 15. The total range of scores possible under this system was from −6 for the worst combination of all factors to 24 for the best possible combination of all factors. Altogether 282 children were given dietary evaluation of this nature. In the final statistical comparison, only the 128 children between the ages of ten to seventeen years were included because the data for those younger contained the results for deciduous teeth as well and hence were uncertain due to the natural loss of these teeth. In the statistical comparison of the average caries figures and average nutrition scores, there was a reduction in dental caries with improved nutrition. This reduction was significant at somewhat in excess of the 2 per cent level.

This is an interesting study, the validity and importance of which hinges on the accuracy of taking of the diet history as well as on the composite nutrition score developed by these authors. J. H. SHAW

Apples and Dental Health. G. L. Slack and W. J. Martin. *Brit. Dent. J.*, 105: 366, 1958.

Few clinical studies have as pleasant a procedure as the current one where fresh rings of apple were provided to the children in the experimental group after each meal and after each in-between meal snack. The varieties of apples used were chosen to provide firm, crisp flesh with easily masticated skins. The test was to determine whether the use of a relatively fibrous foodstuff that required vigorous chewing would alter the incidence of dental caries. At the beginning of the study there were 195 children between six and fifteen years of age in the group receiving the apple rings and 164 children in the same age group in the control group where no special provision of a comparable foodstuff was provided after meals. During the two-year experimental period during which the teeth of the children were examined every six months, the numbers in the two groups dwindled to ninety and eighty-one, respectively. The study was conducted in London and Liverpool and the children were housed in an institution or in other homes where close supervision was possible.

At the end of the two-year period, it was observed that there was less disease of the gums among the children who had received the apple supplement. Fewer children in the group receiving apple had evidence of any disease of the gums and on the average, there was a lesser amount of disease of the gums among the children in this group. These differences were statistically significant. In both the primary and permanent teeth, the average amount of dental caries which developed in the two-year period was less in the group provided with apple rings. Although the differences were not large, they were consistently in favor of the group who received the apple supplement.

The authors justifiably point out that this influence was encouraging although the small numbers of children and a possibly higher susceptibility among the control children makes it necessary to exercise caution in the interpretation of the results. They believed that the action of the apples was twofold: first, the low pH of the apple and the mastication of the fibrous apple particles stimulate the salivary flow, and secondly, the mechanical cleansing action of the apple sweeping over the teeth and gums removes debris and stimulates the tissues of the gum. Altogether, this is an interesting and unique study that needs to be repeated and extended. J. H. SHAW

Experimental Caries with Human Foods. R. R. Steinman, M. G. Hardinge and R. W. Woods. *J. Dent. Res.*, 37: 865, 1958.

Caries-susceptible rats in eight experimental groups were fed various combinations of human foods over a three-generation period. Control rats were fed a high-sucrose purified diet under the same experimental circumstances. Six food groups were described: (1) a basic group composed of fourteen varied foodstuffs; (2) a refined cereal group composed of white enriched bread and refined cornmeal; (3) a whole grain cereal group containing whole wheat bread and unbolted cornmeal; (4) a dessert group containing plain white iced cake and lemon meringue pie; (5) a fruit group composed of apple, orange and banana; and (6) a vegetable category containing carrots, cabbage, apple, orange and banana. All rats in the eight experimental groups received basic food group 1 and one or two other food groups. The desire in these food combinations was to obtain mixtures of human food that would somewhat approximate human dietaries, albeit from a much more restricted choice of foodstuffs than would be typical in the human population. The food components were ground and mixed thoroughly. Each group of rats in the first generation consisted of thirteen subjects; for each of the second and third generations three females and one male were selected to be the parents in each of the subject groupings. In each of the second and third generations, breeding was stopped in each group when thirteen offspring were available for the new generation.

The nutritional status of the rats from one generation to the next was appraised by noting weaning weights, weekly weight gain, total body weight, length of mandible, ability to reproduce and general physical appearance. A value from 1 to 100 was assigned for each of these parameters; the average nutritional state of each group of rats was determined and called the "nutritional index." Caries scores were similarly rated in order to give the "caries index." The exact means by which these indices were determined was not described; their validity and their value were also left in doubt.

The rats fed a mixture of food groups 1, 3 and 6 had the poorest nutritional index throughout all three generations and the highest caries scores in the second and third generations. The rats fed a mixture of food groups 1 and 2, the rats fed a mixture of food groups 1 and 3 and the rats fed the synthetic diet had the best nutritional status and the lowest caries scores. The other groups were intermediate both with respect to nutritional index and caries score. The authors concluded that there was an association between good nutritional status and low incidence of caries. While their average data point strongly toward this conclusion, the question arises as to whether or not the data are based upon sufficient numbers of animals in each generation to merit the confidence placed upon the statistical evaluation. This query appears to be particularly pertinent in view of the lack of any obvious correlation between the content of the major nutrients in the several diets and the actual nutritional status of the offspring. J. H. SHAW

Potentiating Effects of Prenatal X-irradiation on Dental Caries in the Rat. B. H. Ershoff and L. A. Bavetta. *Proc. Soc. Exper. Biol. & Med.*, 97: 202, 1958.

The aim of this investigation was to study whether or not x-radiation during the period of development of embryonal teeth would, by arresting growth of teeth, potentiate the susceptibility of rats to dental caries. One group of female rats of the Long-Evans strain received a single dose of 150 r x-radiation on the tenth, fourteenth or eighteenth day of pregnancy, respectively. The offspring was fed a cariogenic diet composed of 35 per cent skim milk powder, 18 per cent glucose, 45 per cent cornstarch and 2 per cent dehydrated liver with supplements of vitamins A, D_2, and alpha-tocopherol. The offspring of mothers who had received radiation on the tenth day of pregnancy showed a 20 per cent increase in incidence, and a tenfold increase in the severity of dental caries as compared to control animals fed the stock diet. Radiation was less effective if given on the fourteenth day and had no effect if given on the eighteenth day of pregnancy.

M. SILBERBERG

Intraperitoneal Injections of Carbohydrates to Suckling Rats, and Subsequent Dental Caries. R. R. Steinman, M. I. Haley and P. O'Day. *J. Dent. Res.*, 37: 719, 1958.

The influence of the oral administration or the intraperitoneal injection of monosaccharides on the subsequent incidence of dental caries in suckling rats was evaluated in three experiments. The levels of carbohydrate provided were in addition to the nutrients obtained by suckling at will.

In the first trial, one group of nine rats was fed a 20 per cent solution of glucose at a level to provide 1 mg. of glucose three times a day from the third to the twenty-first day of age. A second group of littermates was provided the same amount of glucose through three intraperitoneal injections per day over the same period. The rats at weaning were fed a purified cariogenic diet *ad libitum*. Those rats given the injections had significantly more tooth decay at the end of a thirteen-week assay period than did the rats provided the oral supplement. Unfortunately there was no control group, so these data do not indicate whether greater incidence of caries was caused by these supplements than would have occurred under the control circumstances.

In the second experiment, there were three groups of suckling rats. The first received a 20 per cent solution of an equal mixture of glucose and fructose by injection, a second group received the same solution by oral administration and the third group without carbohydrate supplements served as controls. The same levels of carbohydrate were used as in the first experiment. Both "carbohydrate groups" had significantly greater incidences of dental caries than the control group. It is interesting to note that the control group had an almost indistinguishable incidence of dental caries compared to the group given the carbohydrate injection in the first experiment and a significantly higher incidence than those given the oral carbohydrate supplement. While this comparison is not entirely valid because of the litter differences between experiments, it does point out the necessity for exactness in designed, thoroughly controlled experiments.

In the third experiment, three groups were given injections of a 20 per cent solution with an equal mixture of glucose and fructose at the levels used in the two previous experiments. While the rats in the first group received the carbohydrate injection only, the rats in groups 2 and 3 were provided with either 0.1 mg. of vitamin C per 10 gm. of body weight or 0.1 mg. of vitamin B_6 per 10 gm. of body weight. At the end of the assay period, the rats in groups 2 and 3 had significantly lower incidences of dental caries than the rats in group 1. Unfortunately, again there were no control rats without a carbohydrate supplement and the average incidence of dental caries in groups 2 and 3 was about half of that of the control rats in experiment 2.

While these experiments have interesting applications in the controversial area of carbohydrates and incidence of dental caries, more carefully designed experiments are needed in spite of the problems encountered in such designs.

J. H. SHAW

The Effect of Sugar in Various Forms on the Rate of Spread of Occlusal Caries in the Lower Molars of Rats. G. N. Davies and B. G. Bibby. *J. Dent. Res.*, 37: 570, 1958.

A preliminary "caries-initiation" period was used in this experiment to allow the development of grossly detectable carious lesions. Throughout this period the rats were kept on diet 580 which is composed of fine granulated sugar, 66 per cent; dried skim milk powder, 32 per cent; and desiccated liver, 2 parts. Oral examinations were conducted at weekly intervals until gross lesions could be detected *in vivo* under a twentyfold magnification. When lesions were detected, the location and size were recorded and the rat assigned to one of five groups which received either (1) ration 580 with drinking water; (2) this ration without any fine granulated sugar which was designated as ration 580M along with drinking water; (3) ration 580M plus 10 per cent sucrose solution; (4) ration 580M plus a carbonated beverage; and (5) ration 580M plus reconstituted orange juice. Except for the rats fed ration 580M and water, the amounts of ration and sugar solution, carbonated beverage and orange juice provided were adjusted so that the same quantity of sugar, milk powder and desiccated liver were provided for each animal in each comparison.

At the end of the second phase of the experiment, the "caries-spread" period, the rats fed ration 580 plus water and those fed ration 580M plus the 10 per

cent sucrose solution had had the greatest increase in caries score during the second period of the experiment. The increase was somewhat greater for the rats in the group fed ration 580 plus water, i.e., the sugar in solid form; however, on the basis of the "t" test, this difference was not statistically significant. The rats fed ration 580M plus water and those fed ration 580M plus the carbonated beverage had comparable increases in caries score during the "caries-spread" period, both of which increases were significantly less than the increase experienced by the group fed ration 580 plus water. No statistical comparison of these increases was made with the increase experienced by the group provided ration 580M plus the 10 per cent sucrose solution. In this regard the authors appear to have been remiss as the differences between these three groups appear by inspection to be insignificantly small. The group fed ration 580M plus orange juice had the lowest increases in caries score. Part of this may be attributed to the incomplete consumption of the orange juice aliquots or possibly the actually soluble sugar content of the orange juice may have been less than the estimated value obtained from anthrone determination. However, the reduced increase with orange juice was sufficiently greater than the increases in other groups to be partially unexplainable by the above reservations.

The authors conclude that there was no difference between the effect on the spread of caries of sucrose in aqueous solution and sucrose in solid form and that sugar as present in a carbonated beverage or in an orange juice concentrate was less capable of permitting carious lesions to progress. Possibly additional statistical testing of the results would indicate that only the small increase in caries score produced by the orange juice was significantly different from the increase caused by the 10 per cent sucrose solution.

J. H. SHAW

Calcification. XVI. Composition of Bones and Teeth in Relation to Blood and Diet in the Cotton Rat. A. E. Sobel and A. Hanok. *J. Dent. Res.*, 37: 631, 1958.

This report concerns a continuation of the series of studies by these authors in which strong support was obtained for the hypothesis that there is "a relationship between the inorganic composition of teeth and the fluid from which the tooth salts precipitate and that the composition of this fluid in turn is related to the blood serum."

Three groups of cotton rats were maintained for twenty-eight days after weaning at sixteen days of age on either a stock diet, a high calcium-low phosphorus diet composed of natural foodstuffs with a Ca : P ratio of 10.4 or a low calcium-high phosphorus diet with a Ca : P ratio af 0.093. The cotton rats on the diet with the Ca : P ratio of 10.4 grew poorly and had normal serum calcium levels, low serum phosphate levels, low $PO_4 : 2CO_3$ molar ratios in the enamel and dentin of the molars and incisors and in the femur and tibia. The cotton rats on the diet with the low Ca : P ratio of 0.093 grew slowly and had low serum calcium values, somewhat elevated phosphate values, high $PO_4 : 2CO_3$ molar ratios in the enamel and dentin of the molars and incisors as well as in the incisor and tibia. The Ca : PO_4 molar ratios in the calcified tissues of the rats on the diet with the high Ca : P ratio and in the diet with the low Ca : P ratio were influenced to a much lesser extent and less predictably than the $PO_4 : 2CO_3$ ratios. In general, the Ca : PO_4 ratios of the calcified tissues of cotton rats in the diet with the high Ca : P ratio were increased while those on the diet with the low Ca : P ratio were decreased. In both test cases, the influences on Ca : PO_4 ratios and $PO_4 : 2CO_3$ ratios were greater for bone than for dentin and for dentin than for enamel. A part of this at least is attributable to the fact that relatively little enamel and dentin is formed in the molars, especially during the experimental period.

In another experiment, major differences were observed between the analyses for the cotton rat and those for the common laboratory rat which suggest that strong genetic influences control composition of teeth and possibly limit the extremes that can be produced within a given species by dietary means during development of teeth.

J. H. SHAW

The Effect of Pyridoxine and Injected Carbohydrate on Incidence of Caries, Dentinal Circulation Related to Diet. R. R. Steinman and M. G. Hardinge. *J. Dent. Res.*, 37: 874, 1958.

This series of studies was designed to test whether or not various systemic influences imposed after development of teeth was largely completed would alter the initiation and progression of carious lesions. In the first experiment, one group of littermate rats was fed Purina Laboratory Chow and a second group a cariogenic purified diet. Trypan blue injections were given to determine whether the "circulation" within the dentin was altered by the diets. It was reported that more trypan blue was found in the dentin of the rats fed Purina than in those fed the cariogenic diet. Microscopic examination of the secondary dentin of the rats on the cariogenic diet indicated a series of irregular wavy concentric rings whereas the secondary dentin of the rats fed Purina had a uniformly regular formation. In the second experiment, one group of littermate rats was fed a ration composed of foodstuffs said to be distributed as in a typical American diet; the second group of littermates received the same diet supplemented with 16 mg. pyridoxine per kg. The rats in the second group had 55 per cent less caries than the control animals. This difference was statistically significant with a "P" value of 0.03.

In the third experiment, a group of littermates was fed the same ration as the control rats in the second experiment except that the dessert items had been omitted; the experimental littermates received a solution containing a mixture of equal parts of glucose

and fructose by intraperitoneal injection to provide 1 mg. of carbohydrate per gm. of body weight three times a day. The experimental rats had an average caries score that was 2.9 times greater than the control rats, which constituted a highly significant increase.

Although obviously the data are not strictly comparable from one experiment to another by reason of the different litters represented, the control rats in the second experiment had an average caries score that was 2.3 times greater than that of the control rats in the third experiment despite the same experimental duration and fairly comparable diets except for the dessert category. Thus, the intraperitoneal injection of these amounts of glucose and fructose appeared to be as cariogenic as the oral consumption of the carbohydrates in the desserts. All three experiments were considered to be evidence to support the thesis that the initiation and progression of dental caries are modified by postdevelopmental systemic influences.

J. H. Shaw

Dental Caries in Two Strains of Rats Fed Diets of Two Different Degrees of Fineness. C. D. Miller and C. A. Schlack. *J. Nutrition*, 66: 105, 1958.

Animals of the Navy strain are resistant to dental caries; those of the Hawaii strain are susceptible. Rats of both strains were fed a stock diet for 100 days beginning at the age of twenty-one days. One group was given the diet in coarse form, while the others were fed the same diet ground to a fine meal. At the termination of the experiment the teeth were studied with reference to the number of teeth with caries, involvement of the alveoli and severity of the lesions. Coarse meal protected the teeth even of the susceptible animals, while in the susceptible rats feeding of the fine meal resulted in a significantly higher incidence and greater severity of caries than in the group fed the coarse ration.

M. Silberberg

The Dental Caries Response of Rats Fed Cariogenic and Non-Cariogenic Diets for Different Periods of Time. C. D. Miller. *J. Nutrition*, 66: 113, 1958.

The aim of the experiments reported here was to learn if weanling rats first given a non-cariogenic diet would be protected against later feedings of a caries-producing diet; and whether the effects of feeding first a caries-producing diet would carry over to a later "good-diet" period.

The author used as a non-cariogenic diet one with which there had been long experience: a mixture of skim milk powder, wheat flour, cornmeal, brown rice flour and soybean flour, with added yeast, Wesson oil, cod liver oil and salt mixture. The cariogenic diet was prepared by the introduction of powdered sugar replacing wheat flour to the extent of 18 per cent of the calories. The mixtures were ground to 48-mesh fineness. A strain of rats known to be susceptible to caries was used. The animals were started on the experiment after weaning, at twenty-one days, and after the appearance of the first and second molars, but not the third molars. The observations were continued for 90 to 100 days, with diet changes at about mid-point.

"When the noncariogenic diet was fed for about half the experimental period of 100 days and the cariogenic diet the second half, the teeth were well protected; whereas if the cariogenic diet was fed the first half of the experimental period, the dental conditions were as bad as if the rats had the cariogenic diet for the entire period." The author suggests that the findings may well indicate the danger to children of a poor diet in the early years, especially during the preschool period when the permanent teeth are being formed.

Frank E. Rice

INSULIN, ORAL AGENTS AND DIABETES

The importance of the prediabetic state cannot be overemphasized. In the pregnant patient, recognition and appropriate treatment of pre- or gestational diabetes leads to a marked improvement in fetal salvage. In the older groups, the insidious onset of serious irreversible degenerative changes is forestalled by early detection of the disease. Vigilance and serial testing of diabetes-prone patients is mandatory in the well organized medical practice.

The Late Puerperal Glucose Tolerance of Mothers of Large Infants. P. H. Futcher, A. L. Haslup and B. O. Nugent. *Bull. Johns Hopkins Hosp.*, 102: 235, 1958.

Since the birth of a large infant may indicate that diabetes may later develop in the mother, glucose tolerance tests were performed approximately six weeks after delivery on fifty-five mothers of infants who weighed more than 4,000 gm. at birth. Four of the mothers had abnormal glucose tolerance curves characterized by blood glucose levels in excess of 140 mg. per 100 ml. two hours after ingestion of 100 gm. of glucose. All four of these women were 24 pounds or more overwieght. The authors suggest that these women should be regularly examined and that weight loss should be encouraged as a diabetes-preventive measure.

M. W. Bates

The Prediabetic State in Man. Definition, Interpretation and Implications. J. W. Conn. *Diabetes*, 7: 347, 1958.

In this article, the Banting Memorial Lecture of 1958, the author reviews the background in the development of the cortisone-glucose tolerance test and the experience with it to date. In some 300 relatives of diabetic patients with normal glucose tolerance curves, the cortisone-glucose tolerance test was positive in 25 per cent while it was positive in only 3 per cent of control subjects with no family history of diabetes. This test looks very promising as a method of detecting

the prediabetic state long before clinical diabetes becomes apparent. G. HOLLIFIELD

The study of the mode of action of oral antidiabetes agents has led to a more thorough understanding of certain aspects of insulin action. The slow "physiologic" administration of insulin has been shown to decrease the rate of release of glucose from liver cells. Thus, the suppression of glucose entry to blood from liver obesrved with oral agents, such as tolbutamide or chlorpropamide, may be attributable to release of endogenous insulin into the portal circuit.

Action of Insulin and Tolbutamide on Blood Glucose Entry and Removal. G. Jacobs, G. Reichard, E. H. Goodman, Jr., B. Friedmann and S. Weinhouse. *Diabetes*, 7: 358, 1958.

Patients with diabetes and subjects without diabetes were given a "trace" dose of uniformly C^{14}-labeled glucose and blood samples were removed at frequent and regular intervals before and after administration of insulin or tolbutamide. Before injection of insulin or tolbutamide, the logarithmic drop of specific radioactivity coincident with a constant blood glucose concentration indicated a constant rate of replacement of the blood glucose. The "turnover" rates, at 1 to 2 mg. per 100 ml. per minute were at least as high in the patients with diabetes as in the normal subjects. On intravenous insulin injection, there was an immediate, transient suppression of glucose entry and the removal rate was increased approximately threefold. With intravenous administration of tolbutamide, glucose entry was suppressed as with insulin, but the removal rate was unaffected. This action of tolbutamide was similar to that of subcutaneously injected insulin, which also caused a suppression of blood glucose entry without affecting its removal. The data are in accord with an action of tolbutamide in stimulating secretion of insulin, and are regarded as emphasizing the role of insulin on hepatic glucose output in the physiologic action of the hormone. G. HOLLIFIELD

The Mode of Action of Oral Antidiabetic Compounds. A. Káldor and G. Pogátsa. *Lancet*, 1: 386, 1959.

The action of tolbutamide given with substances of known glycolytic effect was examined in male albino rats. The glycolytic effects of thyroxine, curare and posterior pituitary extract were all inhibited by administration of tolbutamide. Glycolytic action is probably their only similarity, however it may be actuated. Tolbutamide possibly exerts its effect by "impeding glycogenolysis, thus 'defending' the glycogen content of the liver." F. E. HYTTEN

Pharmacology and Mode of Action of the Sulphonylureas in Man. J. M. Stowers, R. F. Mahler and R. B. Hunter. *Lancet*, 1: 278, 1958.

Although considerable clinical experience has now been accumulated on the use of sulphonylureas in diabetes, relatively little is known of the basic pharmacology. Investigations were made in both normal persons and patients with diabetes. Tolbutamide was distributed in a volume corresponding to about 15 to 25 per cent of the body weight, equivalent to the extracellular fluid; carbutamide had about double this volume of distribution.

Carbutamide had a half-life of thirty-three hours in the blood stream; for tolbutamide it was four hours. Tolbutamide should therefore be given at least twice a day. Twenty-four hours after a dose of tolbutamide, from 10 to 28 per cent of it is found in the plasma as the physiologically inactive carboxylic ester. Tolbutamide appears to be excreted by the renal tubules. From studies on patients with diabetes it is concluded that the sulphonylureas significantly reduce the output of glucose from the liver; there was no evidence that they increased secretion of insulin. F. E. HYTTEN

Role of Insulin in Acute Hypoglycaemic Action of Tolbutamide. J. A. Weaver, T. E. Prout, G. W. Scott and S. P. Asper. *Brit. M. J.*, 1: 425, 1958.

The pharmacologic action of the sulphonylureas is not finally settled. The study reported here investigates some of the possible actions in ten patients with recent and untreated diabetes, and in rabbits.

Tolbutamide administration did not affect the arteriovenous glucose difference, indicating that the rate of uptake of glucose by the tissues is not influenced. The insulin-like activity of the serum, assayed by the rat diaphragm method, was unchanged and tolbutamide had been given. The rate of disappearance of intravenously administered insulin labeled with I^{131} was studied in rabbits by an electrophoretic technic. No difference in the rate of disappearance of the labeled insulin was found after intravenous injection of tolbutamide. These results indicate that the hypoglycemic action of tolbutamide is not produced by increasing the effective level of insulin. F. E. HYTTEN

Comparison of Response to Intravenously Administered Sodium Tolbutamide in Mild Diabetic and Nondiabetic Subjects. R. H. Unger and L. L. Madison. *J. Clin. Invest.*, 37: 627, 1958.

Sodium tolbutamide was administered intravenously to 100 normal subjects and seventy-nine patients with mild "stable" diabetes. The blood glucose concentration was determined at twenty-minute intervals after administration. The blood glucose levels of subjects without diabetes reached a nadir between twenty and forty minutes after injection. Patients with diabetes, however, including those with a normal fasting blood glucose concentration, exhibited a more gradual decline.

The response of normal subjects to administration of tolbutamide under these conditions resembled that of a normal insulin tolerance curve. If it is shown that tolbutamide administration produces a release of stored insulin from the pancreas, then the more gradual

hypoglycemic response to tolbutamide administration, which patients with diabetes show, could be ascribed either to diminished insulin stores, a decreased rate of insulin release in response to stimulation or extra-pancreatic interference with insulin degradation.

S. O. WAIFE

Oral Treatment of Diabetes. G. H. Hall, M. F. Crowley and A. Bloom. *Brit. M. J.*, 2: 71, 1958.

A short trial of a new oral hypoglycemic agent (N-beta-phenethylformamidinyliminourea or DBI) is reported. The drug was tried in forty patients with diabetes of varying grades of severity, aged eighteen to seventy-eight years. In doses of 150 mg. DBI caused a fall of blood sugar in about two-thirds of the subjects; the change is apparent after four hours, maximal at about six hours and ceases its effect after ten hours. It had no effect on ketosis. In five cases where tolbutamide proved to be ineffective, DBI produced a fall in blood sugar. Side effects, mainly nausea, vomiting or diarrhea, occurred in twenty-five of the subjects and prevented any trial of prolonged treatment.

F. E. HYTTEN

Preliminary Clinical Observations on the Use of a Biguanide (DBI) as an Oral Hypoglycemic Agent. C. Weller and A. Macaulay. *J. Am. Geriatrics Soc.*, 7: 128, 1959.

Forty-two patients with diabetes who required insulin were given DBI (N-beta-phenethylformamidinylliminourea) for periods of one to six months. Using a decrease in blood sugar level of 30 per cent or more as the criterion, this drug, in varying doses, was effective in 46 per cent of this group. Therapy with DBI was considered successful in twenty-two patients in that they could tolerate it and it replaced insulin entirely in the majority and reduced the insulin requirement in the remainder. The most troublesome side effects encountered were nausea and vomiting.

G. HOLLIFIELD

Mode of Action of Oral Antidiabetic Compounds. K. Lundbaek, K. Nielsen and O. Rafaelsen. *Lancet*, 1: 1036, 1958.

This is a concentrated review (therefore an adequate abstract is impossible) of the present knowledge about the mode of action of the oral antidiabetic drugs. It is obvious that a considerable amount of research has taken place in many countries in the few years these drugs have been available and yet there is no clear understanding of their precise action. The hypoglycemic effect cannot be explained by interference with endocrine balance, but it may be that either depression of liver glucose output or enhanced peripheral glucose utilization occurs; there is no detailed evidence to support either possibility.

"It is suggested that the hypoglycaemic effect of the oral antidiabetic compounds is determined not by the presence or absence of insulin but by the degree of diabetic aberration of metabolism." F. E. HYTTEN

Improvement in the diabetic state to the point of remission for variable, usually brief periods, is not a rare observation. It is seen with a fair degree of frequency in juvenile patients with diabetes whose insulin requirements may decrease strikingly after initial stabilization on insulin and diet.

Remittent Insulin-Insensitive Diabetes. R. Wells. *Brit. M. J.*, 2: 1328, 1958.

A number of pathologic processes and physiologic conditions are known to alter the sensitivity to insulin and in some instances fluctuation occurs without any obvious cause. Eight patients from Singapore are described who exhibited it to an extreme degree.

These eight people, ranging from a sixteen year old girl to a sixty-three year old man, had sudden episodes of insulin insensitivity. Each needed over 200 units of insulin daily for at least seven days and one needed 720 units daily. All had complete or almost complete remissions during which time they needed no treatment whatever. All except two have subsequently relapsed after remissions of from twelve to thirty-eight months. No reason for this behavior is suggested.

It is thought that insulin requirements of patients with diabetes in Singapore is higher than the average in Britain, possibly due to the elimination of insulin-sensitive patients with diabetes during the war-time Japanese occupation.

F. E. HYTTEN

The early experiments of Houssay have been repeated in baboons with essentially the same results. The anti-insulin effects of pituitary extracts and cortisone have been subject to wide study.

Endocrine Control of Blood Sugar, Lipaemia, and Ketonaemia in Diabetic Baboons. J. Gillman, C. Gilbert, E. Epstein and J. C. Allen. *Brit. M. J.*, 2: 1260, 1958.

Five male baboons were totally pancreatectomized and then two to fourteen months later totally hypophysectomized. After hypophysectomy the baboons had a greatly increased sensitivity to insulin and were maintained on from 6 to 10 units daily. They maintained their body weights of 11 to 18 kg.

Deprivation of insulin resulted in a marked rise in blood sugar, comparable to that during insulin deprivation before hypophysectomy. The blood lipids rose (but in two baboons to a lower level than before hypophysectomy), but the blood ketones remained low. Two of the baboons died but there are detailed reports of the effect of hormone administration to the other three.

The administration of cortisone raised the levels of lipemia, cholesterolemia and phospholipemia above those obtainable before hypophysectomy, but the rises were unaccompanied by ketonemia. Administration of a saline suspension of acetone-dried human

anterior pituitary scarcely affected the serum lipids but did produce severe ketonemia and hyperglycemia. The classic complete diabetic picture was reproduced in the depancreatized-hypophysectomized baboons by administration of cortisone in conjunction with the crude pituitary suspension.

The relationship between these and other hormones and diabetes are discussed in some detail. This is an important contribution to the subject.

F. E. HYTTEN

The rise in beta-lipoprotein observed in poorly controlled diabetes may represent the effect of mobilization of depot lipids occurring with the decrease in glucose uptake by fat depots. The beta-globulin lipid-carrier protein elevations noted to rise in protein fractionation studies in patients with diabetes may be found to be a compensatory response to the lipid mobilization.

Serum-Lipids and Blood-Sugar Levels in Childhood Diabetes. O. H. Wolff and H. B. Salt. *Lancet*, 1: 707, 1958.

Since diabetes strongly predisposes to premature atherosclerosis, the study of serum lipids and lipoproteins in patients with diabetes might throw light on the chemical changes which precede the arterial disease. The results of estimates of blood sugar and total lipid, lipoprotein lipid, cholesterol esterified fatty acid and phospholipid in serum, for thirty-five children under "good or fair diabetic control" are presented in some detail. The mean levels of beta-lipoprotein lipid, cholesterol and esterified fatty acid in the serum were significantly higher in the children with blood sugar levels above 200 mg./100 ml. than in those with lower levels. In three untreated patients with only mild ketosis initial serum lipid levels were high, due largely to beta-lipoprotein. Under insulin treatment blood sugar fell, followed within a few days by the level of beta-lipoprotein.

The results "support a hypothesis that hyper-beta-lipoproteinaemia develops whenever insufficient carbohydrate is available for metabolic needs."

F. E. HYTTEN

It appears that the beta cells of the islets can selectively take up glucose and certain other sugars similar to the selective affinity of thyroidal cells for iodide. The oxidation of the sugars evolve such substrates as pyruvate and oxaloacete which may function as chelators for zinc resulting in release of insulin.

Stimulation of Insulin Output by Monosaccharides and Monosaccharide Derivatives. G. Pozza, G. Galansino, H. Hoffeld and P. P. Foa. *Am. J. Physiol.*, 192: 497, 1958.

The ability of various sugars and closely related substances to stimulate insulin secretion was studied by means of pancreatic-femoral cross circulation experiments between hepatectomized donor dogs and normal recipients. In other experiments, the test substance was injected directly into the pancreatic artery of normal dogs. The administration of *d*-glucose, *d*-galactose or *d*-ribose was followed by a prompt hypoglycemia, suggesting insulin secretion; *d*-arabinose caused an unexplained delayed hypoglycemia, while *d*-fructose, *d*-mannose, *d*-xylose, *l*-arabinose, 3-methyl-glucose, *d*-glucosamine, galacturonic acid and saline had no effect. The tentative hypothesis that insulin secretion is stimulated by sugars which are both utilizable and insulin-sensitive is offered. No relationship between chemical structure and ability to cause release of insulin was found. AUTHORS

Evidence for Stimulation of Insulin Secretion by Growth Hormone in the Rat. F. L. Engel, T. Albertson, J. Fredericks and E. Lopez. *Endocrinology*, 63: 99, 1958.

Although there is a moderate amount of indirect evidence to suggest that growth hormone stimulates the secretion of insulin or release of preformed insulin from the pancreas, previous studies in this laboratory were not in accord with this view. The authors feel that negative results obtained in earlier experiments occurred because fasted rats rather than fed animals were used when the effect of growth hormone on glycogen content of adipose tissue was determined. The current experiments were again based on the knowledge that insulin is unique in its ability to increase concentration of glycogen in adipose tissue. Both growth hormone and insulin, given to fed rats following glucose administration, induced significant increases in glycogen content of the dorsal interscapular brown fat pad. Eviscerated rats under similar conditions did not respond when growth hormone was administered. When growth hormone and insulin were injected simultaneously into eviscerated rats only effects of insulin were apparent. These experiments support the view that growth hormone stimulates secretion or release of insulin from the pancreas but it is pointed out that growth hormone also has other effects on carbohydrate metabolism.

A. B. EISENSTEIN

Insulin-binding antibodies found in the gamma globulins of most subjects treated with insulin has excited much investigation concerning the role of these factors in the phenomenon of insulin resistance in patients with diabetes. The following papers deal further with this problem.

The Role of Nonprecipitating Insulin Antibodies in Diabetes. J. H. Skom and D. W. Talmage. *J. Clin. Invest.*, 37: 787, 1958.

Antihuman gamma globulin was prepared in rabbits. This was added to an appropriate soluble mixture of an insulin-resistant patient's serum and I^{131}-labeled insulin. A precipitate would then result. Supernatants were analyzed electrophoretically on a starch block. By this method it was possible to measure the amount of

binding of insulin by the sera of forty-eight subjects. Insulin binding was significant in the sera of all twenty-three patients who had received insulin recently for more than one month. On the other hand, twelve control sera from patients who had never received insulin were negative. However, in contradiction to the recent reports of Berson and Yalow (*Diabetes*, 6: 402, 1957; *J. Clin. Invest.*, 36: 873, 1957) and Burrows, Peters and Lowell (*J. Clin. Invest.*, 36: 393, 1957), no consistent relationship could be demonstrated between the insulin binding and the insulin requirements of patients receiving insulin. Failure of insulin antibodies to accelerate insulin metabolism and a reversibility of the insulin-antibody bond are possible explanations by the authors for the relative lack of effect of antibodies on the insulin requirements of the patients.

A recent pointed editorial by Berson and Yalow (*Am. J. Med.*, 25: 155, 1958) discusses insulin "antagonists," insulin antibodies and their relationship to insulin resistance. Although the antibody nature of particular insulin antagonists has been established, they can be clearly differentiated from others that are demonstrable in the absence of previous administration of exogenous insulin. However, antibody or non-antibody antagonists are not proved to be causal factors of diabetes. After a decade of study of insulin antagonists, it is hoped that some "antiantagonist" therapy or "desensitization" regimen will evolve of clinical value.

E. COHEN

The Effect of Insulin-Binding Antibodies on Insulin Sensitivity. N. Kalant, C. Gomberg and R. Schucher. *Lancet*, 2: 614, 1958.

Using I^{131}-labeled insulin it has been shown that insulin is bound to plasma globulins and disappears slowly from the circulation in persons previously treated with insulin; specific antibodies have been postulated. In this study, patients with diabetes who were previously treated with insulin had a lesser insulin response than patients with diabetes who had no treatment, but the response was not apparently related either to the degree of globulin binding or to the degree of insulin retention in the plasma.

Experiments with rabbits are reported in detail. Repeated injection of insulin led to the development of hemagglutinating antibodies to insulin; the removal of insulin from the circulation was altered and it was shown to be attached to β-globulin. There was no evidence of a decrease in the hypoglycemic response to insulin or of the presence of anti-insulin activity of the serum.

Infusion of serum obtained from insulin-treated patients with diabetes into normal rabbits led to the globulin-binding of insulin but did not alter the rate of insulin removal. It is concluded that insulin-binding antibodies and hemagglutinating antibodies are not responsible for decreased responsiveness to insulin or for its slower removal from the circulation.

F. E. HYTTEN

Insulin Antagonism in Plasma of Diabetic Patients and Normal Subjects. J. Vallance-Owen, E. Dennes and P. N. Campbell. *Lancet*, 1: 336, 1958.

It has been shown previously in this laboratory that some diabetic plasma contains an insulin antagonist, demonstrable *in vitro*. There is also evidence that it exists, but is inhibited, in normal persons. In this paper, experiments are described of attempts to isolate the antagonist. It resides in the albumin fraction of the plasma but it is not known whether the antagonism is due to albumin itself or to a substance tightly bound to it. The antagonistic activity of the albumin in whole normal plasma is counteracted by some other substance which may be insulin itself.

F. E. HYTTEN

Variability of insulin clearance rates from different injection sites and from an area of lipodystrophy produced by repeated injection is an important clinical phenomenon. Delay in absorption may result in diabetic acidosis in the extreme case.

Rate of Clearance of Insulin Labelled with I^{131} from the Subcutaneous Tissues in Normal and Diabetic Subjects. C. L. Joiner. *Lancet*, 1: 974, 1959.

The suggestion has been made that local atrophy or hypertrophy of fat at the site of insulin injections might be associated with poor control of diabetes. In this study the clearance of insulin labeled with I^{131} from subcutaneous injection sites was studied in fifty-nine male and thirty-eight female control subjects and twenty-four male and seventeen female patients with diabetes.

In male control subjects the half-period of clearance was 67 $\pm$ 11 minutes from the arm, 123 $\pm$ 18 minutes from the leg and 76 $\pm$ 20 minutes from the abdomen; the equivalent figures for females were 55 $\pm$ 7, 99 $\pm$ 12 and 76 $\pm$ 20, respectively. Variation in volume or concentration of the injection, and in the amount of exercise taken by the subject, had no significant effects on the rate of clearance. Hyaluronidase increased absorption rates.

In the group with diabetes, slow clearance from leg sites was most common in women, but where clearance from the leg was slow, it might be normal in the arm. Lipohypertrophy or lipoatrophy was found in nineteen of the patients with diabetes and was associated with poor clearance. The importance of varying the site of injection is stressed.

F. E. HYTTEN

My personal experience (CRS) with salicylates in patients treated with insulin has not proved satisfactory. No sustained reduction in insulin dosages has been observed unless anorexia and reduced food intake occurred with salicylate administration.

The Insulin Equivalence of Salicylate. J. Reid and T. D. Lightbody. *Lancet*, 1: 897, 1959.

It has been recently noticed that salicylate has an antidiabetic effect. In this study the amount of

insulin which could be replaced by a maximum tolerated course of aspirin was investigated in a group of fourteen patients with diabetes. The subjects were aged thirty-three to seventy-four years. Nine were women in whom the duration of diabetes varied from one to twenty-four years and the insulin requirement varied from 12 to 112 units per day.

It was possible to reduce the insulin dosage in every case after two to three weeks' treatment with aspirin. Eight subjects with an initial requirement of 12 to 48 units were able to do without insulin altogether and blood sugar levels indicated stable control; another five patients had a reduction of from 10 to 20 units but labile blood sugar levels indicated that they were poorly controlled with either treatment. The other subject had an initial requirement of 112 units reduced to 72 units. Broadly speaking, the higher the insulin requirement the greater was the amount of hormone which could be replaced by aspirin. According to the authors "these results place salicylate in the class of promising oral anti-diabetic substances, and should stimulate investigations to remove its undesirable effects and to find out how it acts." F. E. HYTTEN

Enhanced Sugar Uptake Fails to Simulate the Insulin Effect on Lipogenesis in the Isolated Perfused Rat Liver. D. E. Haft and L. L. Miller. *Am. J. Physiol.*, 193: 469, 1958.

Large quantities of glucose and fructose were infused without insulin into isolated livers of fasted normal and fed rats with diabetes as a test of the widely accepted theory that the single primary role of insulin is to speed the entry of glucose into the cell. The liver disposed of large quantities of hexose in most cases without simultaneously increasing, as with insulin, the rate of incorporation of acetate-1-C^{14} into fatty acids. Much of the sugar was transformed to glycogen and CO_2 without entering the pathways of fatty acid or ketone body synthesis, or influencing the conversion of acetate to these products. With livers of fasted normal rats, massive fructose infusion significantly inhibited urea formation and reduced the incorporation of acetate into glucose and plasma protein. The dissociation between carbohydrate balance and lipogenesis suggests that insulin has some primary action other than making more sugar available in metabolism. It is suggested that there is an anatomic separation of metabolic pathways in the liver, and that insulin serves to facilitate glucose transport between them.

AUTHORS

IRON METABOLISM

The average normal diet contains 12 to 15 mg. of iron of which perhaps 5 to 10 per cent is absorbed replacing the daily average non-hemoglobin loss of about 1 mg. Nutritional iron deficiency develops easily in menstruating and childbearing women and in growing children. When iron intake is excessive, iron appears to remain in a slowly moving pool, entering the liver gradually as it exceeds the amount which can be used by the bone marrow. Deposition of iron in liver cells is conditioned by the nutritional status, increasing in the presence of protein deficiency.

Serum Iron Levels in Siderosis Due to Habitually Excessive Iron Intake. J. Higginson, K. J. Keeley, M. Andersson and A. R. P. Walker. *J. Clin. Invest.*, 36: 1723, 1957.

The serum iron level was studied in a series of twenty-six South African Bantu adults and one child. The results were correlated with the severity of siderosis and determined by biopsy of the liver. The serum iron levels in subjects in whom the deposit of hemosiderin was absent or minimal were definitely lower than those in subjects with heavily siderotic livers. However, once significant iron deposits were present in the liver, no correlation was apparent between the degree of hemosiderin deposit and mean serum iron levels.

S. O. WAIFE

Experimental Nutritional Siderosis. T. Gillman, P. A. S. Canham and M. Hathorn. *Lancet*, 2: 557, 1958.

There are two opinions currently held about the cause of the commonly found severe siderosis in Africans. One believes that it is due to iron "overload" alone; the other that malnutrition is also involved. In this study male rats were fed on normal stock ration or uncooked "mealie-meal," half of each group also receiving 4 per cent ferric citrate as an addition. Biopsies of the liver were carried out at 50 to 100 days and the rats analyzed at 156 days. Briefly, the results showed that although iron uptake was increased in both diets when iron was added, the body iron in the rats fed mealie-meal was about double that of those who were well fed, due largely to greatly increased iron in the liver. In early degrees of siderosis the excess iron in the liver was confined to the liver cells themselves; only in the advanced condition did the sinusoidal and portal phagocytes become laden, possibly due to ingestion of effete liver cells. These results support the view that excess iron intake alone does not lead to siderosis if the basal diet is good.

F. E. HYTTEN

Experimental Dietary Siderosis. T. Gillman, M. Hathorn and P. A. S. Canham. *Am. J. Path.*, 35: 349, 1959.

Male rats of the Wistar strain weighing 200 to 230 gm. were fed one of the following diets *ad libitum* for twenty-two weeks: (1) a stock diet (77 mg./100 gm. iron content); (2) "mealie," an uncooked corn meal diet (13 mg./100 gm. iron); (3) the stock diet enriched with iron (1,017 mg./100 gm. iron); and (4) the "mealie" diet enriched with iron (974 mg./100 gm. iron). The mean daily iron intake and the mean iron absorption were as follows: in group 1, 18 and 0.19 mg.;

in group 2, 0.6 and 0.26 mg.; in group 3, 203 and 0.35 mg.; and in group 4, 107 and 0.57 mg., respectively. The iron content of the liver in mg./100 gm. wet weight was 32.3 mg. in group 1; 22.4 mg. in group 2; 66.6 mg. in group 3; and 367 mg. in group 4. The uptake of iron from the iron-enriched "mealie" diet was thus much higher than that from the iron-enriched stock diet. The iron pigmentation of the tissue set in earlier and was more intense than in the former group. Spleen, lymph node and bone marrow became siderotic at an earlier experimental stage; in the liver iron was deposited in the parenchymatous cells before it was demonstrable in the portal and sinusoidal phagocytes. The colon was heavily laden with iron, but the duodenum was practically free of it. Pancreas, salivary glands and kidney tubules showed pigment only at a late stage. Dietary siderosis is thus not due to simple overloading of iron, but there should be in addition a disturbance of the intracellular metabolism due to malnutrition.

M. SILBERBERG

Excessive Iron Absorption in Rats Fed Low-Protein, High-Fat Diet. N. Kaufman, J. V. Klavins and T. D. Kinney. *Lab. Invest.*, 7: 369, 1958.

Young male albino rats of the Sprague-Dawley strain weighing about 220 gm. were fed one of the following rations for several months: (1) 10 per cent protein, 13 per cent carbohydrate and 60 per cent lard; (2) diet No. 1 with a supplement of 2 per cent iron citrate; (3) 18 per cent protein, 55 per cent carbohydrate and 10 per cent lard; (4) diet No. 3 with a supplement of iron citrate; (5) control rats restricted in their food intake to that of group 1; or (6) to that of group 3. Other minerals and vitamins were available in adequate amounts. The tissues were studied and analyzed chemically with reference to the deposition of iron. The total content of iron in liver and pancreas was markedly increased only in animals fed the low protein, high fat ration.

M. SILBERBERG

The absorption of iron is incompletely understood. It is believed that iron enters mucosal cells of the upper small intestine and combines with a protein to form ferritin. The presence of ferritin is thought to block further iron uptake until the iron complex delivers its iron component to the blood stream where it combines with an iron-binding globulin, transferrin. This concept does not receive support in some studies.

Absorption of Inorganic Iron from Graded Doses: Its Significance in Relation to Iron Absorption Tests and the "Mucosal Block" Theory. M. D. Smith and I. M. Pannaccuilli. *Brit. J. Haemat.*, 4: 428, 1958.

Considerable evidence has been published which suggests that in the animal the absorption of iron from the intestine is reduced after large doses because of a "mucosal block." There have been fewer reports for man.

In this experiment graded amounts of ferrous sulphate (from 0.001 to 300 mg. of iron), labeled with Fe^{59}, was given to forty-eight subjects without anemia and seven patients with iron deficiency anemia. Absorption was calculated by estimating fecal loss.

The mean absorption in the normal subjects fell from 33 to 12.6 per cent as the dose rose; in the anemic subjects it fell from 50 to 37.5 per cent. No limit of absolute iron absorption was reached and there was considerable individual variation. "The results indicate that mucosal control on the absorption of soluble inorganic iron is slight and does not limit absorption to body requirements."

F. E. HYTTEN

A Quantitative Study of the Fate of Recently Absorbed Food Iron. C. Neerhout, H. Armstrong, J. Schulz and J. Smith. *Am. J. Dis. Child.*, 95: 126, 1958.

This study was undertaken to determine the amount of Fe^{59} detectable in the blood and tissues of normal, iron-deficient and iron-loaded dogs fourteen days after the administration of isotopically labeled food iron. Six healthy mongrel dogs were used. Two were fed a standard ration with daily supplements of horse meat and a polyvitamin preparation. Two received a similar diet plus intravenous injections of saccharated iron oxide (Proferrine®) for twenty-seven days. The iron-deficient animals received a milk diet with supplemental cooked polished rice as needed to control weight loss and diarrhea. Percutaneous femoral vein phlebotomies were also carried out until reticulocytopenia persisted in the face of profound microcytic hypochromic anemia (twenty-seven and thirty-five days, respectively). The radioactive test meals fed to the animals consisted of Fe^{59}-tagged chicken liver and eggs.

The results of the metabolic balance studies at the end of the fourteen-day experimental period revealed that from 96 to 103 per cent of the orally administered dose was recovered in the stool, blood and tissues of the animals. Of the iron which was absorbed, 95 per cent or more was present in the circulating hemoglobin mass in all animals. The amount of iron absorbed was generally proportional to the state of iron deficiency. The iron-loaded animals not only absorbed slightly more iron than the control animals but proceeded to incorporate more into the hemoglobin. A measurable amount of Fe^{59} activity was detected in all tissues of iron-deficient animals. In the iron-loaded and control animals this was limited mainly to the liver and the spleen. Of interest is the deposition of iron in both soluble and insoluble forms by the iron-loaded animals.

Thus, iron absorption proceeds as well, if not better, in the face of increased iron stores as in normal animals. This suggests that either there is no effective control of iron entrance to the body or the regulatory mechanism is lacking in some siderotic subjects. The widespread deposition of Fe^{59} in the iron-deficient dogs suggests a generalized tissue deprivation of this substance and points out the fact that the anemia is only the most easily demonstrable manifestation of a widespread iron deficiency.

T. C. PANOS

A Quantitative Study of the Absorption of Food Iron in Infants and Children. J. Schulz and J. Smith. *Am. J. Dis. Child.*, 95: 109, 1958.

Iron deficiency is the most common nutritional deficiency encountered in children and is almost exclusively due to inadequate dietary intake of iron. Balance studies were conducted on 126 children to determine the amount of food iron absorbed and that utilized for hemoglobin production. The quantity of radioiron absorbed was considered to be that amount of the ingested iron which did not appear in the feces. The amount of the ingested iron absorbed in use for hemoglobin production is that quantity of the administered iron isotope which was present in the circulating red blood cells two weeks after the tagged-iron feeding.

It was found that normal infants and children absorb an average of about 10 per cent of the iron naturally occurring in cow's milk, eggs and chicken liver and of iron supplements added to commercially prepared infants' cereals. The observed values for iron absorption in children were consistently higher than those reported for adults, in the case of milk and eggs. Children with iron deficiency absorbed two to three times as much food iron as did normal children. Normal children absorbed more iron from milk than did normal male adults. Children less than three years of age absorbed more iron from eggs than did children three to ten years of age. The absorption of food iron is more closely related to age and serum iron levels than the hemoglobin values or red cell indices. Anemic children with iron depletion absorb and utilize much larger amounts of iron than normal control subjects. Whereas the absorption of iron seem to be temporarily impaired in infants with diarrhea, there were no significant differences in iron absorption in several children with a variety of illnesses (chicken pox, otitis media, infection of the respiratory tract and leukemia). To date, no child was observed in whom impairment of the iron-absorption mechanism was demonstrable. Lack of hematologic response is not adequate evidence for inability to absorb orally given iron.

Dietary and iron can satisfy the iron requirements of selected normal infants on an optimal diet, but the selected infants are probably not representative of a large segment of the population. T. C. Panos

Quantitative Study of the Absorption of Iron Salts in Infants and Children. J. Schulz and J. Smith. *Am. J. Dis. Child.*, 95: 120, 1958.

Because iron supplementation of many infants' diets is often desirable, balance studies utilizing a radio-iron salt (ferrous sulfate) were made in thirty-four normal and five iron-deficient infants and children, with particular reference to the influence of certain liquids and the size of the iron dose on the absorption of the iron salts. A ferrous salt was selected because of the published reports that bivalent salts are better assimilated than trivalent ones. Tracer doses of Fe^{59} sulfate were added to a commonly used pharmaceutical preparation (Fer-in-Sol®) one, two or four times daily one hour before a meal and at least two hours after ingestion of other food. The tracer dose of radioactive iron did not add significant amounts of elemental iron to that usually present in the preparation used. The largest single dose of ferrous iron tolerated and absorbed was 30 mg. Twelve to 15 per cent of the 30 mg. doses of Fe^{++} given once or twice a day were absorbed by normal children. In both normal children and those with iron-deficiency anemia, ferrous sulfate was less well absorbed when added to milk or orange juice than when given alone. Under all circumstances, infants with iron deficiency absorbed more ferrous iron than do normal infants.

Therapeutic iron is indicated only if specific evidence of iron deficiency exists. Since body iron other than hemoglobin iron is deficient in these patients, storage and tissue iron, as well as hemoglobin iron, must be replenished before therapy is terminated. This ordinarily should not be less than one month and frequently should be longer than one month, in dosages in the range of 75 mg. of elemental iron in three or four divided doses. T. C. Panos

The vasodepressor and antidiuretic action of ferritin may be a significant factor in certain malnutrition states.

Ferritin in Nutritional Oedema. S. G. Srikantia. *Lancet*, 1: 667, 1958.

The iron-protein complex, ferritin, has a potent antidiuretic effect and has been reported in the blood stream of patients suffering from a number of edematous states. In five of six children with kwashiorkor and two adults with nutritional edema, ferritin was found in the blood; it disappeared after recovery. It is suggested that ferritin may be associated with oliguria and edema in these states, and may have been released to the blood stream by damage to the liver.

F. E. Hytten

A reduction in hemoglobin values below 12 gm. during pregnancy is very commonly encountered. This may represent the effect of dilution rather than iron deficiency. However, until the point is entirely cleared, the administration of iron to pregnant women should be recommended.

The Prophylaxis of Iron-Deficiency Anaemia in Pregnancy. D. N. S. Kerr and S. Davidson. *Lancet*, 2: 483, 1958.

A retrospective study of 1,994 case notes of women pregnant during 1955 and 1956 was made to study the incidence of anemia. It is concluded that "mild anemia," a hemoglobin concentration of below 12.6 gm./100 ml. is common in early pregnancy and there is the usual finding of a decreasing hemoglobin concentra-

tion as pregnancy progresses. The incidence of "anemia" was not associated with age, parity or the number of pregnancies within the past five years.

An experiment was conducted to compare the effects of ferrous gluconate and ferrous sulphate in doses of 105 mg. of iron daily. There was no difference apparent, either in potency or side effects; the addition of ascorbic acid to ferrous gluconate made no observable difference. Dietary surveys conducted by questionnaire concluded that the mean daily iron intake in pregnant women from Edinburgh was about 13 mg. These findings are now well known and have led the authors to the conventional conclusion that pregnant women should be given iron routinely; they are apparently not aware of substantial arguments to the contrary. F. E. HYTTEN

There are many iron preparations on the market containing copper, cobalt, molybdenum or vitamins, all of which are relatively expensive and none of which has been proved superior to iron alone. There are relatively few patients who cannot tolerate iron therapy or who are unable to absorb it due to gastrointestinal disease. In these conditions, parenteral administration of iron should be employed.

Gastrointestinal Intolerance to Oral Iron Preparations. D. N. S. Kerr and S. Davidson. *Lancet*, 2: 489, 1958.

Ferrous sulphate, ferrous gluconate, ferrous succinate, ferrous calcium citrate and lactose-containing control pills were adminstered to ninety-three healthy young women in a double-blind trial. Each subject took all five types of pill (one containing 35 mg. Fe three times daily) for periods of five days and noted any symptoms; a break of two days was made between trials of different pills. There was no difference in the incidence of symptoms.

In a second experiment control pills were given to two groups; that the pill was inert was known to one group; the other group was not aware of this. There was a much lower incidence of symptoms in the group with the "unknown" control pills and it is concluded that the intolerance to iron pills, in the amounts given, is largely psychologic in origin. [This is a rather striking paper and should be read in the original—Ed.] F. E. HYTTEN

A Comparative Study of the Use of Ferric Chelate in Iron-Deficiency Anaemia. C. F. Herridge. *Brit. M. J.*, 2:140, 1958.

A newly introduced iron compound—the ferric sodium salt of ethylenediamine tetra-acetic acid—has been compared with ferrous gluconate and succinate in a group of mentally-defective hospital patients with anemia. There was a somewhat greater rise of hemoglobin concentration in those subjects having the chelated iron but this may have been due to a higher absolute iron dosage.

Side effects, particularly diarrhea, were more frequent and more severe with the chelated iron and there was a small prolongation of the prothrombin time associated with its use.

Over all, it seems to be a hematinic with no advantages and several disadvantages, compared to those more commonly used. F. E. HYTTEN

Iron Metabolism in the Pregnant Rabbit: Iron Transport Across the Placenta. T. H. Bothwell, W. F. Pribilla, W. Mebust and C. A. Finch. *Am. J. Physiol.*, 193: 615, 1958.

Studies on iron metabolism were made in pregnant rabbits. The amount of iron transported from the maternal plasma to the fetus increased progressively with age and weight of the fetus. By the end of the pregnancy 90 per cent of the plasma iron turnover was directed to the fetus. The majority of fetal iron is deposited as non-hemoglobin iron. Transport of placental iron occurs against a concentration gradient. The uptake of iron by the placenta is an active process independent of the fetus, and retrograde transfer of iron from the fetus to the mother does not occur. Studies of altered maternal metabolism indicate that placental transport reflects both fetal demands and the state of iron metabolism in the mother. AUTHORS

Iron-59 Metabolism as an Index of Erythropoietic Damage and Recovery in Monkeys Exposed to Nuclear Radiations. Q. L. Hartwig, G. S. Melville, Jr., T. P. Leffingwell and R. J. Young. *Am. J. Physiol.*, 196: 156, 1959

Monkeys exposed to ionizing nuclear radiations were studied for changes in radioiron utilization by erythrogenic tissues. Twenty-eight hours after exposure, $Fe^{59}Cl_3$ was injected; twenty-four hours later the first blood sample was drawn. Iron incorporation by the animals exposed to radiation indicated marked erythropoietic depression. At fifty-two hours after exposure, average uptake in the animals exposed to radiation was 2.1 per cent as compared to 10.7 per cent in the control animals. Only three of the original ten animals who received radiation survived. On repeating Fe^{59} injection in supplemented groups of survivors thirty-four days after exposure, repair and active erythropoiesis were indicated. Plasma iron exhibited periods of both hyper- and hypoferremia. Those animals who died were consistently hypoferremic for two to three days prior to death. AUTHORS

ITEMS OF GENERAL INTEREST

Dietary Studies on Salivary Protease Activity in Caries-Susceptible Rats. J. B. Resnick, N. P. Willett and J. H. Shaw. *J. Nutrition*, 66: 411, 1958.

A strong correlation exists between caries susceptibility and the protease activity in mixed saliva of the Hunt-Hoppert strains of caries-susceptible and caries-resistant rats. However, no comparable correlation

exists in the Harvard strains of caries-susceptible and caries-resistant rats. The present study was conducted to determine whether wide variations in the carbohydrate-protein-fat ratios of the diets on which the Hunt-Hoppert and Harvard caries-susceptible rats were maintained would alter the level of protease activity in the saliva and whether complete prevention of the initiation of carious lesions would reduce the salivary protease levels.

Three diets were used in each of the two caries-susceptible strains: The first was a highly cariogenic diet containing a high amount of sucrose, little fat and a normal level of protein, the other two diets contained high levels of protein and fat and no carbohydrate. In both strains, the cariogenic diet caused a high incidence of carious lesions, while the second and third diets did not permit the initiation of any carious lesions. Irrespective of the diet fed and irrespective of the presence of carious lesions, the salivary protease activity in all three groups in each strain was not altered by the experimental procedure but remained at levels typical of the strain: high for the Hunt-Hoppert caries-susceptible strain and low for the Harvard caries-susceptible strain.

These dietary studies strengthen the hypothesis that the salivary protease activity is a genetically determined trait and is not secondary to the development of carious lesions or to an increased oral flora by reason of the presence of carious lesions. J. H. SHAW

Influence of the Salt Mixture Added to Cariogenic Diets of Comparable Sucrose Content. J. Haldi, W. Wynn, J. H. Shaw and R. F. Sognnaes. *J. Nutrition*, 66: 333, 1958.

The observation that the purified cariogenic diet prepared and used in the Emory laboratories was much less cariogenic than the highly comparable purified diet containing the same amount of sucrose used in the Harvard laboratories has already been reported (*J. Nutrition*, 50: 267, 1953).

The present study was conducted partly in the Emory laboratories and partly at Harvard to determine what part of the diets was responsible for the striking difference in cariogenicity. Similar studies were conducted in the two institutions. The principal experiments involved the interchange of the salt mixtures in the Emory and Harvard diets so that four diets were tested: (1) the Emory diet containing the Emory salt mixture; (2) the Emory diet containing the Harvard salt mixture; (3) the Harvard diet containing the Harvard salt mixture; and (4) the Harvard diet containing the Emory salt mixture. The composition of the Emory salt mixture was based on the original Osborne-Mendel mixture while the Harvard mixture is a modification of the Phillips-Hart formula. In the Emory laboratories, the rats fed the Emory diet with the Emory salt mixture (diet 1) had the lowest incidence of dental caries while the rats fed the Harvard diet with the Harvard salt mixture (diet 3) had the highest incidence of dental caries. Incorporation of the Harvard salt mixture into the Emory diet (2) increased the incidence of carious lesions while the incorporation of the Emory salt mixture into the Harvard diet (4) decreased the incidence of carious lesions. The data from the Harvard experiments were not as consistent or as striking, but in general the trend was in the same direction.

The salt mixtures appear to differ in regard to their influence upon the initiation and progression of dental caries in the rat. The inconsistencies in the results between various experiments suggest that the influence of the salt mixtures on the cariogenicity of the diets is of such a subtle nature that it may be overwhelmed by presently unrecognized factors. These results clearly emphasize the complexity of the dental caries problem.

J. H. SHAW

The Caries-inhibiting Effect of Dibasic Sodium Phosphate and Dibasic Calcium Phosphate Added to Wheat Flour and Bread Diets. F. J. McClure and A. Muller. Jr. *J. Am. Dent. A.*, 58: 36, 1959.

The addition of various inorganic phosphates to cariogenic diets has become of considerable interest because of the ability of some dietary phosphates to alter the initiation and progression of carious lesions by striking amounts.

In the present paper, dibasic sodium phosphate and dibasic calcium phosphate were tested either in the diet, in the drinking water or through their incorporation into wheat flour to be used in preparation of bread dough with the bread later used in cariogenic diets. The addition of a 2 per cent supplement of dibasic sodium phosphate to the diet caused major reductions in the severity of dental caries in two different strains of rats. The addition of 2 per cent dibasic sodium phosphate to the drinking water was ineffective for the more caries-resistant of the two strains but was almost as effective in the more caries-susceptible of the two strains as the dietary supplement had been. Supplements of 1.4 or 1.5 per cent dibasic calcium phosphate or of 1.25 per cent calcium carbonate to the diet had little or no influence on the severity of carious lesions. When 2 per cent dibasic sodium phosphate was incorporated into wheat flour for the preparation of bread to be used in the cariogenic diet, a major reduction in dental caries was caused, although not quite as great as when this compound was incorporated directly into the diet. However, when 2 per cent dibasic calcium phosphate was incorporated into wheat flour for the preparation of bread, it proved to be as effective as the addition of dibasic sodium phosphate to the bread.

The authors postulated that in the baking process the dibasic calcium phosphate had reacted with the sodium chloride in the dough to become more soluble and hence more effective in the prevention of dental caries. The authors point out the possibility that addition of inorganic phosphates to flour might influence the initiation and progression of caries in human beings and also that self-rising flour already contains approximately 2 per cent monobasic calcium phosphate.

J. H. SHAW

Wheat Cereal Diets, Rat Caries, Lysine and Minerals. F. J. McClure. *J. Nutrition*, 65: 619, 1958.

The investigation of carious lesions on the smooth surfaces of the molars of the laboratory rat has progressed much more slowly than comparable studies on carious lesions that develop in the sulci of the molars. A specific series of experimental conditions, including a suitable diet and a suitable strain of rats, seems to be necessary for the production of this type of lesion. McClure appears to have had a greater success in combining the necessary experimental conditions than any other investigator.

In the current series of studies he has reported on the influence of heating cereal diets, of lysine supplements and inorganic phosphates on the incidence of smooth surface lesions. When diets that contained wheat flours of different origin, dry or toasted bread, or shredded wheat biscuit, cerelose or lactose and liver powder were fed, a high incidence of severe smooth surface carious lesions resulted. When whole wheat flours were autoclaved with cerelose or lactose their caries-producing potential was greatly increased while the toasting of bread was not effective. In all cases, supplementation of the cereal diets with 2 per cent lysine resulted in striking reductions in the incidence of smooth surface lesions. The addition of 2.9 per cent ash of powdered skim milk, of 1.1 per cent calcium carbonate and 0.6 per cent dibasic sodium phosphate or of 1.6 per cent dibasic sodium phosphate alone caused significant reductions of 56 per cent to 80 per cent in carious lesions on the smooth surfaces. However, the addition of either 1 per cent calcium carbonate or 2 per cent dibasic calcium phosphate did not cause any reductions in this type of carious lesion. Analyses of the ash, calcium and phosphorus contents of the dentin and enamel of incisor and molar teeth was unaffected by a moderate deficiency of dietary calcium or by a low phosphorus content. Hence these analytical data appeared to be unrelated to the influences on smooth surface lesions. J. H. SHAW

Dental Caries in the Albino Rat in Relation to the Chemical Composition of the Teeth and of the Diet. IV. Variations in the Ca/P Ratio of the Diet Induced by Changing the Calcium Content. J. Haldi, W. Wynn, K. D. Bentley and M. L. Law. *J. Nutrition*, 67: 645, 1959.

How much dietary calcium and phosphorus independently affect formation of teeth and development of caries, and how important the ratio of one to the other may be, are questions that have not been entirely answered. In these experiments all diets contained a constant percentage of phosphorus, 0.5 per cent. The calcium content was varied as follows: (1) 0.29 per cent; (2) 0.57 per cent; (3) 1.01 per cent; and (4) 1.57 per cent by the addition of calcium carbonate. The ratios of Ca/P in these four diets were considered to be 1:2, 1:1, 1:0.5, and 1:0.3, respectively. The diets were high in carbohydrate, containing 64 per cent sucrose. The protein was casein, 20 per cent. The diets contained 8 per cent fat, 4 per cent yeast and other usual supplements.

Sialoadenectomized rats were used in the observations on formation of caries. In the studies on the effect of diets on composition of teeth, the salivary glands were left intact. In the experiment on caries, all four diets were fed. In the experiments on composition of teeth only diets (1) and (4) were used.

The incisors and the molars were analyzed separately. Separate analyses were also made of the dentin and the enamel.

There was no difference in the composition of the teeth of the animals whether fed diet (1) or (4).

A progressive decrease in cariogenicity occurred from diets (1) to (3). There was no difference between (4) and (3). It is concluded that changes in cariogenicity observed from feeding these diets must be attributed to the actual calcium and phosphorus content and not to the Ca/P ratio.

The authors note that other investigators have concluded that there may be a relation between composition of teeth and the Ca/P ratio of the diet. They suggest that if that hypothesis is correct, "it is applicable only under certain conditions which did not prevail in our experiments." FRANK E. RICE

Studies on the Relation of Dairy Products to Dental Caries in Caries-Susceptible Rats. J. H. Shaw, B. J Ensfield and D. H. Wollman. *J. Nutrition*, 67: 253, 1959.

The object of this study was to determine the influence of milk and milk products, including those containing added carbohydrate, when used as supplements to a diet known to be cariogenic; the influence of diet was measured by the number of carious molars, the number of carious lesions and the extent of carious lesions in the growing rat. Six hundred sixty-nine rats of a caries-susceptible strain were used in the experiment.

The effects of the different diets were observed during two periods: (1) feeding the mother before birth and during the lactation period, and up to the time of eruption of the teeth of the young—"developmental influence"; (2) feeding the young after the teeth had erupted—"posteruptive influence." The different dairy supplements were: (1) whole milk; (2) chocloate milk containing 3.09 per cent fat and 19.66 per cent non-fat solids; (3) a chocolate drink with less fat and solids; (4) a mixture of milk, ice cream and cheddar cheese; and (5) a mixture of chocolate drink, ice cream and cheddar cheese. There were numerous crossings of groups and subgroups of rats from one feeding regimen to another. The control ration was one known to be cariogenic.

Substituting the control ration with any of the dairy supplements at levels of 30 to 45 per cent of the daily caloric intake, when fed to the young during the posteruptive period, significantly reduced the susceptibility

to dental caries. The differences between the various dairy product mixtures were trivial, with some slight apparent advantage in mixtures containing whole milk rather than chocolate milk.

The influence on the teeth of the young from feeding the control ration to the mothers, with and without dairy product supplementation, throughout pregnancy and lactation ("developmental period") was found to be insignificant.

The author questions how closely these findings can apply to man until comparably controlled clinical studies are conducted. FRANK E. RICE

Effects of Drinking Solutions Containing Metallic Ions Above and Below Hydrogen in the Electromotive Series on Dental Caries in the Syrian Hamster. J. Wisotsky and J. W. Hein. *J. Am. Dent. A.*, 57: 796, 1958.

These studies were conducted to determine the influence on dental caries in the Syrian hamster of certain metallic ions that are above hydrogen in the electromotive series (lithium, potassium, magnesium, lead and cadmium) and of others that are below hydrogen in the series (palladium, platinum and gold). For purposes of comparison it was thought that all metallic ions should be compared at the same equivalent concentration in the experimental drinking water solution. The level desired was 1 mEq. per L., but in the case of lead, cadmium and gold, the 0.5 mEq. level was tried to avoid toxicity. However, in the case of cadmium, the concentration had to be further reduced to 0.25 mEq. per L. The anion or negative radical component of the salt used was chosen to provide as soluble a salt as possible. Bromides, nitrates, iodides and phosphates were avoided because of suggestions of potential influence on dental caries.

Among the metallic ions in the series above hydrogen in the electromotive series, the monovalent cations of lithium and potassium had no influence on dental caries at the level tested. The bivalent cation of magnesium significantly accelerated dental caries in the male and female hamster, while lead accelerated dental caries significantly in the male but had no significant influence in the female.

Among the metallic ions in the below hydrogen area of the electromotive series, palladium and cadmium did not significantly affect the caries scores. Platinum caused extreme acceleration of the initiation and progression of carious lesions. Gold caused a strong inhibition of dental caries. Copper has been previously shown to inhibit formation of caries in the Syrian hamster at levels of approximately 1 mEq. per L.

From these data the investigators concluded that the position of a metallic ion in the electromotive series is not an accurate prediction of how that ion will affect formation of dental caries. They also concluded that strong caries inhibition is not a common property of metallic ions but may be confined to certain groups, such as the gold-silver-copper group. J. H. SHAW

Dental Caries in the Syrian Hamster. VIII. The Induction of Rampant Caries Activity in Albino and Golden Animals. P. H. Keyes. *J. Dent. Res.*, 38: 525, 1959.

The Syrian hamster has been a potentially useful subject in investigations on dental caries, but its successful use has been hampered by intermittent breeding problems, susceptibilities to intercurrent infections and unpredictable ability to produce carious lesions.

In the present paper a regimen is described whereby normal breeding has been obtained on a stock diet composed of equal parts of two different commercial diets supplemented with apples, carrots and kale twice a week to stock females and daily to pregnant and nursing hamsters. After weaning a semi-synthetic diet is fed which is composed of 59 per cent confectioner's sugar, 27 per cent skim milk powder, 6 per cent whole wheat flour, 3 per cent alfalfa powder, 2 per cent 1:20 liver concentrate, 2 per cent whole liver powder and 1 per cent Wesson's salt mixture. This diet, when supplemented weekly with apples, carrots and kale, was capable of producing grossly detectable carious lesions in thirty-five days in albino hamsters and forty-two days in golden hamsters. At these ages after consumption of the cariogenic diet, the teeth are heavily covered with food debris and plaque material which made an accurate *in vivo* scoring of the lesions impossible. When the stock diet was fed for seven to twenty-one days after the caries initiation period, the teeth were freed of the debris and any softened carious material was quickly worn away so that easy and accurate caries scores could be made. On the basis of these scores, selections could be made of breeders for maintenance of the colony. In addition, the early onset of caries which was grossly discernible in the upper second molars as early as ten or twelve days after weaning and prior to the eruption of the third molar made possible manipulations to test the pre-eruptive and posteruptive effects of potentially caries-inhibiting agents.

On the basis of the 2,000 hamsters which formed the background of this study, males had a somewhat higher caries activity than females and albinos a somewhat higher activity than golden hamsters. The rate of weight gain appeared to be unrelated to the rate of initiation of carious lesions. Equal caries activity was observed in small litters as in larger ones. The postulate is presented that the offspring may obtain their caries-producing flora from the "cariogenic" flora maintained in the alimentary tract of the mothers. J. H. SHAW

Relationship Between Maternal Ingestion of Carbohydrate in the Rat and Dental Caries Susceptibility of the Progeny. B. F. Watson and J. C. Muhler. *J. Dent. Res.*, 38: 618, 1959.

A comparison of the influence of a stock ration composed of natural food products versus a semi-purified diet when fed during pregnancy and lactation upon the caries susceptibility of the young was made. The stock

ration was composed largely of yellow corn grits, ground yellow corn and powdered whole milk which, on the basis of average food composition values for these materials, would supply about 49 to 50 per cent of starches and dextrines and about 11.5 per cent of lactose. The semi-purified diet contained 60 per cent of sucrose. The latter diet permitted very poor breeding and from sixteen breedings only nine pups were weaned. Four of these were continued on this semi-purified diet for 100 days and had no evidence of gross carious lesions. The other five were transferred to the stock diet and had a low incidence of carious lesions in the same period.

From the sixteen breedings of females on the stock diet, fifty-four pups were weaned. Twenty-five of these were maintained on the semi-purified diet and had an extremely low incidence of carious lesions. Twenty-four were maintained on the stock diet and had a high incidence of carious lesions. Because of the low ability to raise young on the semi-purified diet, a group of the females which had been on this diet for several weeks continued to receive the same diet except that the 5 per cent cottonseed oil was replaced by the same amount of corn oil. Under these circumstances five breedings resulted in forty-two weanlings, a substantially better rate than for the stock diet. Twenty of these rats were continued on the semi-purified diet and a low incidence of carious lesions developed. Another twenty were transferred to the stock diet and a high incidence of carious lesions developed.

Under the conditions of this experiment, the semi-purified diet had a lesser ability postdevelopmentally to cause carious lesions than did the stock diet; in addition, this particular semi-purified diet during pregnancy and lactation did not increase caries susceptibility of the offspring beyond that of this particular stock diet.

J. H. Shaw